Université de Sherbrooke

Identification of Cellular Targets of the Adenovirus E1B 55-kDa Protein

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Abbreviations

3-AT - 3-amino-1,2,4-triazole

5-FOA - 5-fluoroorotic acid

aa - amino acid(s)

Ad - adenovirus

AD - activation domain

Amp - ampicillin

APL - acute promyelocytic leukemia

bp - base pair(s)

BD - binding domain

BLAST - basic local alignment search tool

DAPI - 4',6'-diamidino-2-phenylindole

HAT - histone acetyl transferase

HDAC - histone deacetylase

kb - kilo base pair(s)

kDa - kilo Dalton

NLS - nuclear localization signal

ORF - open reading frame

PBS - phosphate-buffered saline

PI - propidium iodide

POD - PML oncogenic domain

RT - room temperature

SD - synthetic dropout

UAS - upstream activating sequence

Ub - ubiquitin

wt - wild type

Abstract

The adenovirus (Ad) E1B 55-kDa protein is vital to viral growth and complete transformation of adenovirus-infected cells. The detailed mechanisms of E1B action are not yet clear. In order to shed light on the functions of this viral oncoprotein it is essential to determine its cellular targets. The yeast two-hybrid method was utilized to screen the *Saccharomyces cerevisiae* genomic library and the human Hela cDNA library with the Ad2 E1B 55-kDa protein as bait. Partial screening of the *S. cerevisiae* genomic library (10^6 transformants) has revealed several yeast proteins that bind to E1B. Among these are: UFD1, involved in the ubiquitin fusion degradation pathway, a major pathway for selective protein degradation in eukaryotes, RIS1, a member of the SWI/SNF2 family of DNA-dependent ATPases, and Bdf2 which contains two copies of the evolutionarily conserved bromodomain, homologous to the C-terminal half of mammalian $TAF_{II}250$. Human proteins positive for Ad2 E1B 55-kDa binding were identified through partial screening of the Hela cDNA library (7×10^5 transformants). Among them are: UbcH7, a ubiquitin-conjugating enzyme involved in the ubiquitin-mediated degradation of the tumor suppressor protein p53, known to be stabilized and inactivated by Ad E1B 55-kDa, SUMO-1, a ubiquitin-like protein which covalently modifies a limited number of proteins in a manner similar to ubiquitination, ubiquitin itself, and DAXX, implicated in Fas-induced apoptosis.

The identification of a DAXX/Ad E1B 55-kDa interaction has interesting implications for the role of E1B in cell transformation. The pro-apoptotic ability of DAXX appears to be dependent on the physical interaction of DAXX with a tumor suppressor protein, PML. The binding of DAXX by PML results in the localization of DAXX to nuclear bodies, known as PML oncogenic domains (PODs). Ad E1B 55-kDa binds to the C-terminus of DAXX, required for PML-binding and POD localization. Immunofluorescence co-localization experiments establish that in cell lines expressing Ad E1B 55-kDa proteins there is disruption of PML-DAXX association. There is also disruption of POD formation in these cells. These observations suggest that another mechanism by which the Ad E1B 55-kDa protein contributes to cell transformation may be by interfering with an apoptotic pathway that acts through DAXX.

Key words: adenovirus, E1B 55-kDa, oncoprotein, yeast two-hybrid, DAXX

Abstrait

La protéine d'adénovirus (Ad) E1B 55-kDa est essentielle à la croissance virale et à la transformation complète des cellules infectées par adénovirus. Les mécanismes détaillés de l'action d'E1B ne sont pas encore bien connus. Afin déterminer les fonctions de cette oncoprotéine virale, il est essentiel de déterminer ses cibles cellulaires. La méthode de deux-hybride de levure a été utilisée pour cribler la bibliothèque genomique de *Saccharomyces cerevisiae* et la bibliothèque d'ADNc Hela humaine avec la protéine d'Ad2 E1B 55-kDa. Le criblage partiel de la bibliothèque genomique de *S. cerevisiae* (10^6 transformants) a indiqué plusieurs protéines de levure qui se lient à E1B. Parmi elles: UFD1, impliqué dans la voie de dégradation de fusion par ubiquitination, une voie importante pour la dégradation sélective des protéines dans les eukaryotes, RIS1, une membre de la famille SWI/SNF2 d'ADN-dépendente ATPases, et Bdf2 qui contient deux copies du bromodomaine, domaine hautement conservé durant l'évolution et qui est homologue à la moitié C-terminale de TAFII250 de mammifères. Un criblage partiel de la bibliothèque d'ADNc de Hela humaine (7×10^5 transformants) a permis d'identifier des protéines humaines positives pour l'association avec Ad2 E1B 55-kDa. UbcH7 est l'une d'entre elles. UbcH7 est une enzyme d'ubiquitination-conjugaison, impliquée dans la dégradation du suppresseur de tumeur p53, celui-ci reconnu pour être stabilisé et inactivé par Ad E1B 55-kDa. Il y a aussi ubiquitine elle-même, SUMO-1, une protéine semblable aux protéines d'ubiquitination, qui modifie d'un nombre limité de protéines par un procédé similaire à l'ubiquitination, et DAXX, impliquée dans l'apoptose induite par Fas.

L'identification d'une interaction entre DAXX et Ad E1B 55-kDa propose des implications intéressantes pour le rôle d'E1B dans la transformation de cellules. Les capacités pro-apoptotiques de DAXX semblent dépendre de l'interaction physique de DAXX avec une suppresseur de tumeur, PML. La liaison de DAXX par PML provoque la localisation de DAXX dans des corps nucléaires, connus sous le nom de domaines oncogènes de PML (PODs). La liaison d'Ad E1B 55-kDa à la portion C-terminale de DAXX est requise pour la liaison a PML et la localisation dans les PODs. Les expériences de co-localisation par immunofluorescence ont démontré que dans des lignées de cellulaires exprimant les protéines Ad E1B 55-kDa il y a dissociation du complexe PML-DAXX. La formation des PODs dans ces cellules est aussi perturbée. Ces observations suggèrent un autre mécanisme par lequel la protéine d'Ad E1B 55-kDa contribuerait à la transformation cellulaire: celles-ci pour interférer avec un processus apoptotique qui agit par l'intermédiaire de DAXX.

Mots clés: adénovirus, E1B 55-kDa, oncoprotéine, deux-hybride de levure, DAXX

Introduction

General Background on Adenoviruses

Adenoviruses (Ad) are a broad family of viruses with double-stranded DNA genomes of ~35 kilo base pairs (kb). There are greater than 40 adenovirus serotypes classified into subgroups based on their percentage of G + C in DNA, hemaglutination properties, and tumorigenicity (listed in table 1). Adenovirus infections are quite common although most are asymptomatic. They have been associated with respiratory illness, conjunctivitis, and gastroenteritis in children. Adenoviruses are lytic in their natural host but are included within a heterogeneous group of DNA tumor viruses for their ability to induce tumors in experimental systems. Other DNA tumor virus families include hepatitis B viruses, simian virus 40 (SV40) and polyomavirus, papillomaviruses, herpesviruses, and poxviruses. These are listed in table 2 along with their associated cancers. Certain DNA tumor viruses have been directly implicated in human malignancies, such as human papillomavirus type 16 (HPV-16) and HPV-18, the causative agents of cervical cancer and other anogenital cancers. Like adenoviruses, polyomavirus and SV40 are not natural transforming agents. However, these viruses are useful for studying the transformation process. The first report of a pathogenic human virus that could cause cancer in animals was the discovery that adenovirus type 12 could induce malignant tumor formation in rodents (Trentin et al., 1962). This sparked extensive study into the mechanisms of transformation by adenoviruses and other viruses. The application of molecular biology to research on the relationship between DNA tumor

viruses and cancer has led to the identification of different cellular tumor suppressors, contributing to the understanding of human cancer development.

Table 1: DNA tumor viruses and associated cancers.

VIRUS FAMILY	ASSOCIATED CANCERS
Hepadnaviruses	Hepatocellular carcinoma
Polyomaviruses	Various solid tumors
Papillomaviruses	Papillomas and carcinomas
Adenoviruses	Various solid tumors
Herpesviruses	Lymphomas and carcinomas
Poxviruses	Mixomas and fibromas

Modified from: T. Benjamin and P. K. Vogt. 1991. Cell transformation by viruses. *In:* B. N. Fields, D. M. Knipe, R. M. Chanock, M. S. Hirsch, J. L. Melnick, T. P. Monath, and B. Roizman, eds. Fundamental Virology, second edition. New York: Raven Press 291-341

Table 2: Classification schemes for human adenovirus.

SUBGROUP	HEMAGLUTINATION GROUPS	SEROTYPES	ONCOGENIC POTENTIAL		PERCENTAGE OF G + C IN DNA
			Tumours in animals	Transformation in tissue culture	
A	IV	12, 18, 31	High	+	48-49
B	I	3, 7, 11, 14, 16, 21, 34, 35	Moderate	+	50-52
C	III	1, 2, 5, 6	Low or none	+	57-59
D	II	8, 9, 19, 37, 10, 13, 15, 17, 19, 20, 22-30, 32, 33, 36-39, 42	Low or none	+	57-61
E	III	4	Low or none	+	57-59
F	III	40, 41	Unknown		

Modified from: M. S. Horwitz. 1991. Adenoviridae and their replication. *In:* B. N. Fields, D. M. Knipe, R. M. Chanock, M. S. Hirsch, J. L. Melnick, T. P. Monath, and B. Roizman, eds. Fundamental Virology, second edition. New York: Raven Press 291-341

Adenovirus-Induced Cell Transformation

Normal cells maintain strict control of cell growth and differentiation, governed by the requirements of the organism. In response to adverse stimuli, such as environmental stress, DNA damage, and viral infection, the products of tumor suppressor genes can signal growth arrest or programmed cell death. In contrast, transformed, immortalized cells undergo continuous, unregulated growth. All adenovirus serotypes are capable of transforming cells in tissue culture. The transforming genes of adenoviruses, like other DNA tumor viruses, are uniquely viral. Cellular equivalents of these genes do not exist, in contrast to retroviruses, the only RNA viruses with oncogenic potential, which either integrate near a cellular oncogene or carry one in their genomes. Adenovirus oncogenes are expressed early, prior to the onset of viral DNA replication. They encode proteins that interfere with the actions of cellular tumor suppressor proteins and at various points in signaling pathways of apoptosis. This results in the deregulation of cell growth control.

Proteins Involved in Adenovirus-Mediated Cell Transformation

Complete transformation by adenoviruses requires the early region E1 that covers two transcription blocks, E1A and E1B (figure 1). E1A proteins (overlapping proteins of 26- and 32-kDa) force cells to enter S phase by targeting the retinoblastoma tumor suppressor protein, pRb. The E1B 19-kDa protein is a homologue of Bcl2 and

prevents viral-induced apoptosis. The oncoprotein that is the focus of this research is the large adenovirus E1B protein. The E1B 55-kDa protein inhibits the normal biological functions of p53 (Marcellus et al., 1996; Steegena et al., 1995; Yew and Berk, 1992). Figure 2 illustrates the functional domains of E1B 55-kDa including the p53 binding domain. Other viral oncoproteins that interfere with p53 functions include the SV40 large T antigen (Farmer et al., 1992) and E6 of human papillomavirus (Scheffner et al., 1990). p53 is a potent tumor suppressor found mutated in more than 50% of human cancers. In response to various stimuli, p53 up-regulates genes involved in growth suppression at G1 and G2. A major component that contributes to p53-induced cell cycle arrest is p21, a cyclin-dependent kinase (CDK) inhibitor. Inhibiting CDK prevents pRb phosphorylation and leads to growth arrest at the G1/S boundary. p53 can also promote apoptosis by up-regulating proteins such as Bax. The E1B 55-kDa protein associates with p53 and interferes with p53 transactivation of its target genes (Sarnow et al., 1982; Yew and Berk, 1992). It may inhibit p53 function by more than one mechanism. First, the carboxyl-terminal region of E1B 55-kDa seems to act as a direct transcriptional repressor that could be coupled to p53 (Martin and Berk, 1998). Second, E1B 55-kDa specifically inhibits acetylation of p53 by P/CAF at lysine 320 (Liu et al., 2000). P/CAF acetylation of the p53 C-terminus stimulates its sequence-specific DNA-binding ability (Gu and Roeder, 1997).

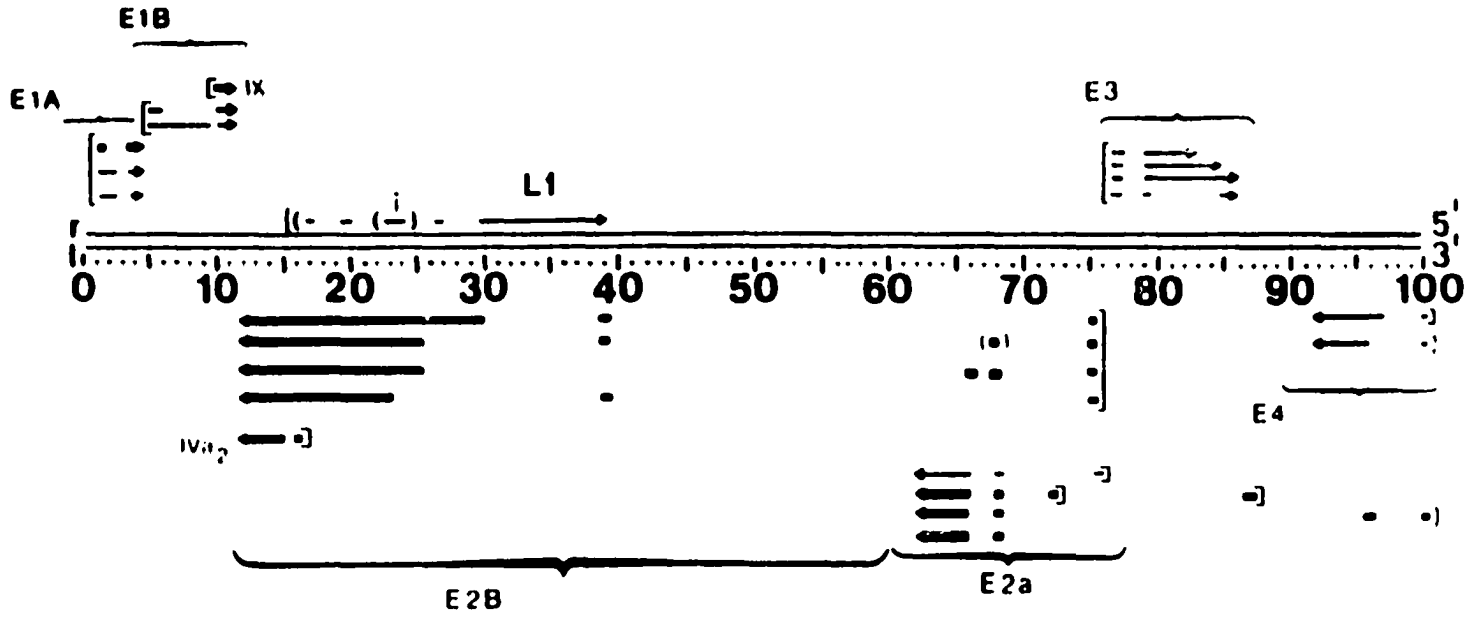

Figure 1: Ad2 genomic map of the early proteins and their mRNAs.
Thin lines indicate mRNAs detected at early times post-infection in the absence of protein synthesis. Thick lines represent intermediate mRNAs most easily detected at late times. Arrowheads show the 3' end. Tentative promoter sites are indicated by [-. *From:* M. S. Horwitz. 1991. Adenoviridae and their replication. *In:* B. N. Fields, D. M. Knipe, R. M. Chanock, M. S. Hirsch, J. L. Melnick, T. P. Monath, and B. Roizman, eds. Fundamental Virology, second edition. New York: Raven Press 291-341

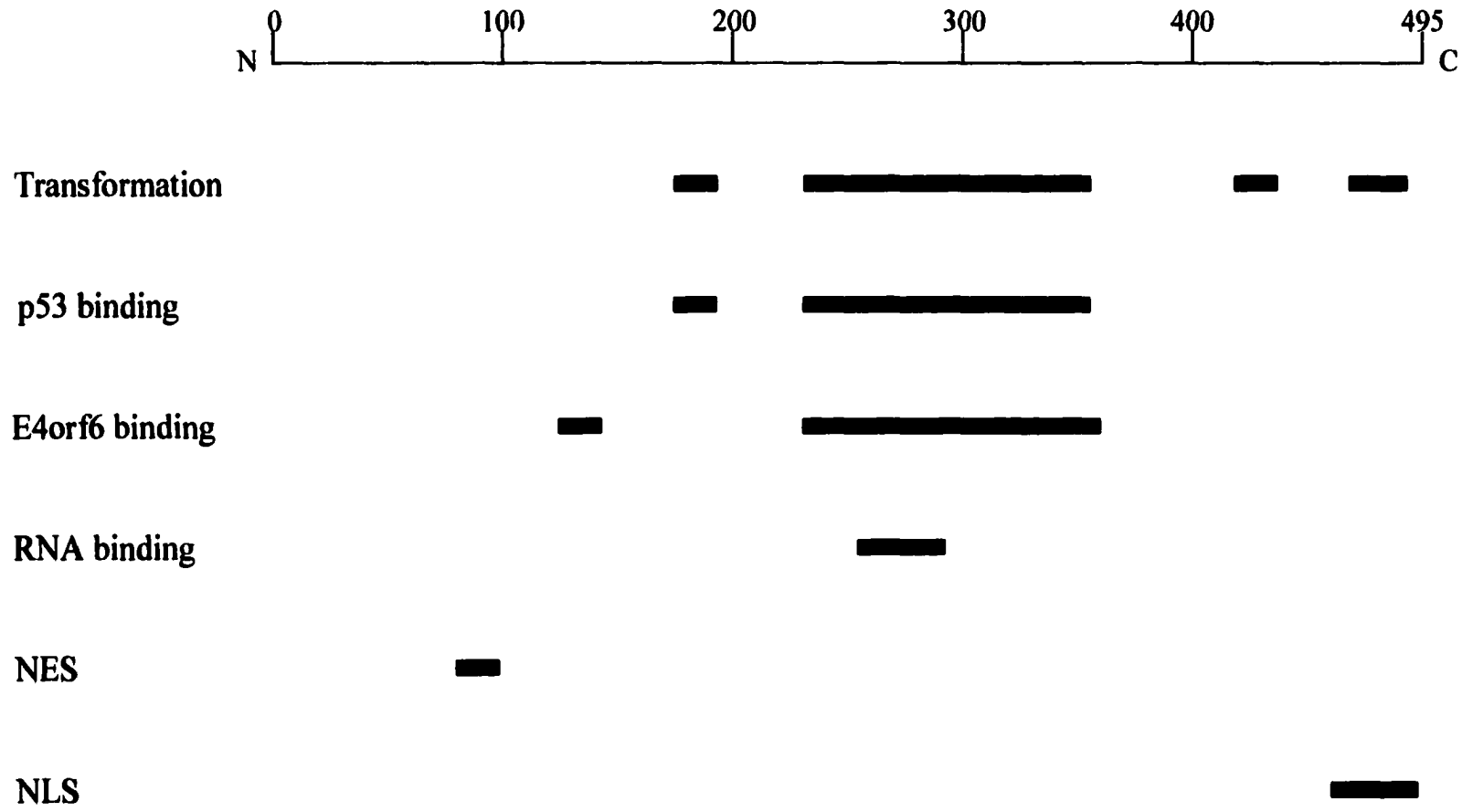

Figure 2: Schematic diagram illustrating the functional domains in the Ad E1B 55-kDa protein. E1B 55-kDa consists of 495 amino acids, represented above. Black rectangles represent known functional domains. NLS, nuclear localization signal; NES, nuclear export signal.

Acknowledging the contribution of p53 inactivation to adenovirus-mediated cellular transformation, there is evidence that points towards mechanisms of E1B 55-kDa action that do not involve p53. This includes experiments performed by Goodrum and Ornelles (1997; 1998) with Ad5 E1B 55-kDa mutants. The presence or absence of p53 did not predict the ability of the cell line to support growth of the E1B 55-kDa mutant virus. In the absence of a correlation between mutant virus replication and p53 status, it is possible that there are other cellular regulatory factors that promote virus replication in the wild type adenovirus infection. Additionally, expression of the tumor suppressor protein, PML, inhibits Ad5 E1A/E1B-mediated focus formation of primary rat cells suggesting that modulation of PML activity plays a role in viral transformation (Nevels et al., 1999).

Research Objectives

The major objective of this research is to identify cellular proteins that bind to Ad E1B 55-kDa. If we take examples from other DNA tumor viruses, we see that their oncoproteins are multifunctional but contain no intrinsic enzyme activity. These proteins exert their effects through binding to numerous cellular proteins. E1B 55-kDa is also a multifunctional protein. Aside from the role that E1B 55-kDa plays in the transformation process, it plays an important role in viral growth. E1B 55-kDa mutations that result in its instability hinder viral DNA synthesis (Mak and Mak, 1990). It is also required for the selective transport of viral mRNA from the nucleus to the cytoplasm (Leppard and

Shenk, 1989). Late viral mRNAs are differentially exported by a protein complex that includes E1B 55-kDa and E4orf6. It has been suggested that the viral RNA transporter of this complex is E4orf6 (Dobbelstein et al., 1997). Recently, however, it has been shown that E1B 55-kDa itself is a good candidate for an adenoviral transporter. It actively shuttles in virus-infected cells (Dosch et al., 2001), with a C-terminal nuclear localization signal (NLS) as well as a N-terminal leucine-rich nuclear export signal (NES) (Krätzer et al., 2000), as first suggested by Liao et al. (1999). E1B 55-kDa also has RNA-binding activity. It contains a domain at residues 249-288 homologous to the RNP domains that mediate direct interaction with RNA (Horridge and Leppard, 1998). It is clear that E1B 55-kDa plays many roles in viral replication; however, the detailed mechanisms of its action are not yet fully understood.

With all of the knowledge that has accumulated on adenoviruses, surprisingly few E1B 55-kDa cellular targets have been identified. Recent reports on associating proteins include PCAF (Liu et al., 2000), and histone deacetylase (HDAC) 1 (Punga and Akusjärvi, 2000). If its binding partners are resolved, this could lead to elucidation of the functions of E1B 55-kDa. The intended focus is E1B-binding proteins involved in cell cycle regulation or apoptosis in order to investigate E1B 55-kDa action in cell transformation. Determining how the targeting of cellular proteins by E1B 55-kDa contributes to transformation by adenovirus may provide new directions for understanding normal cell growth control and cancer development.

Experimental Approach

The yeast two-hybrid method was employed to find Ad E1B 55-kDa interacting proteins. With the use of the two-hybrid method, expression libraries can be screened for interaction with a protein of interest, as illustrated in figure 3. The two-hybrid method is a sensitive means of detecting protein-protein interactions, measured by the re-assembly of a functional transcription factor in yeast, which in this case is Gal4. The Gal4 DNA-binding domain (BD) is separated from the Gal4 transcription activation domain (AD) and each can be fused to a protein. The BD recognizes specific upstream activating sequences (UAS) in the promoter of Gal4 target genes, while the AD stimulates transcription initiation from the UAS by directing the assembly of the transcription complex. For this particular screen, Ad2 E1B 55-kDa is fused to the Gal4 BD and this is referred to as the bait hybrid. A protein encoded by a library insert from either the *Saccharomyces cerevisiae* genomic library or the human Hela cDNA library is fused to the Gal4 AD and this is referred to as the prey hybrid. If E1B 55-kDa binds to a library protein, the Gal4 BD and AD are brought into close proximity and functional Gal4 is generated. This leads to transcription of reporter genes downstream of the UAS. If the two proteins do not interact then the reporter genes are not transcribed. In the host yeast strain utilized there exist three reporter genes: *his3*, *ade2*, and *lacZ*. This allows for nutritional selection (*his3*, *ade2*) as well as color based assays (*lacZ*). A different Gal4 promoter drives each reporter gene. The *his3* reporter provides the highest sensitivity for growth selection and the *ade2* promoter is the most stringent. The numbers of false

positives are greatly reduced with this host strain when compared to earlier strains containing a single promoter element (James et al., 1996).

After determining which cellular proteins associate with Ad E1B 55-kDa through library screening, these interactions can then be verified by alternative means such as immunoprecipitation, GST pull down and immunofluorescence co-localization experiments. The yeast two-hybrid method can be employed to map the domains responsible for the interactions and identify possible E1B-binding domains. Further experiments can then be performed to determine whether E1B 55-kDa interferes with the cellular functions of each positive protein. For example, E1B-binding may prevent the protein from interacting with other cellular proteins or block domains required for its activity or cellular targeting. Approaches to studying biological significance would be determined based on the protein of interest.

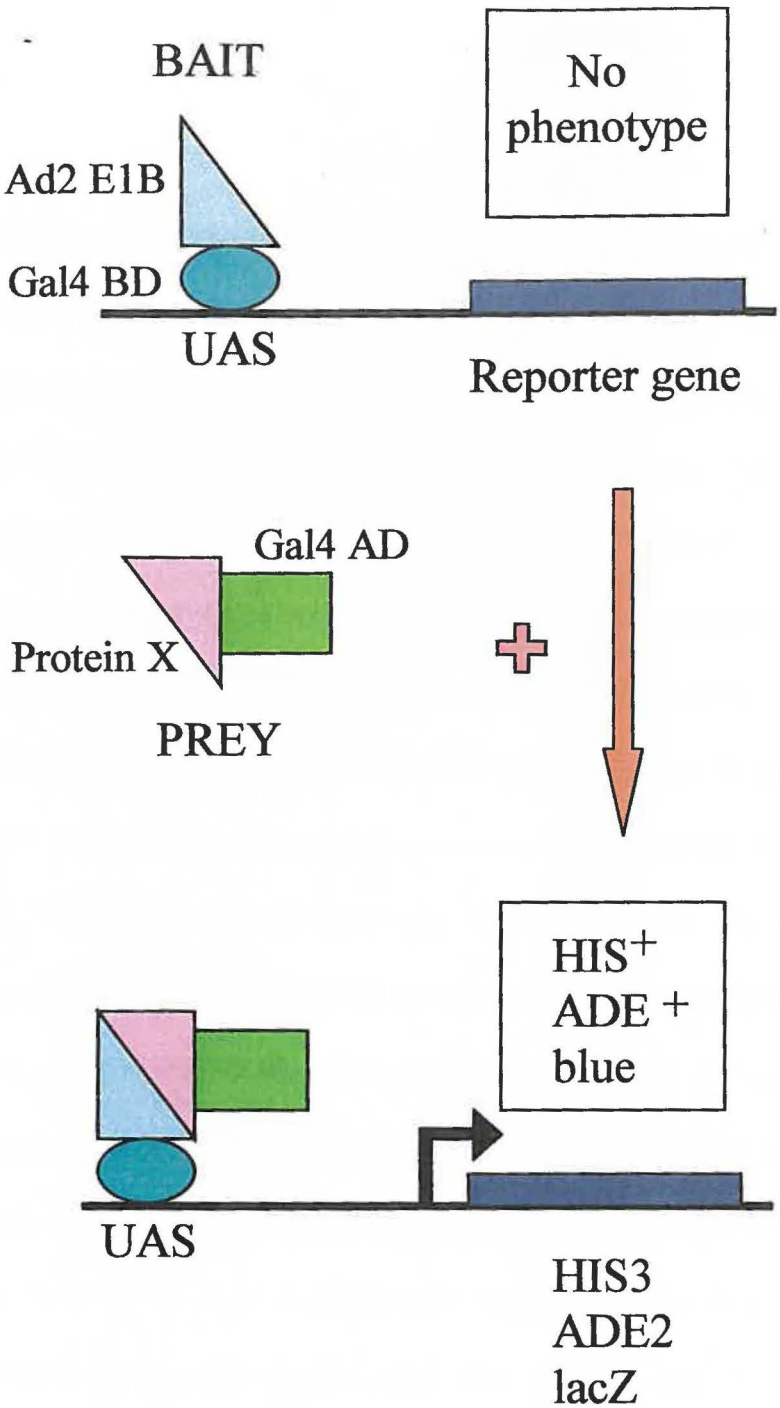

Figure 3: Scheme of the yeast two-hybrid system used for library screening. Bait: Ad2 E1B 55-kDa fused to the Gal4 DNA-binding domain (BD). Prey: human HeLa cDNA or *S. cerevisiae* genomic DNA library protein (protein X) fused to the Gal4 activation domain (AD). In the absence of an interaction between Ad2 E1B 55-kDa and protein X, neither the bait nor the prey hybrid can activation transcription of reporter genes.

Expected Results

If the human adenovirus oncoprotein does behave as oncoproteins of other DNA tumor viruses, then screening the Hela cDNA library with E1B 55-kDa as bait should yield a large number of interacting proteins. These proteins are expected to be involved in nucleocytoplasmic transport, transcription, and cell cycle regulation. A drawback to screening the Hela cDNA library is the amount of false positives that would be encountered. False positives would consist of human proteins associating with either the Gal4 UAS or the Gal4 BD irrespective of the presence of E1B 55-kDa. Performing the same yeast two-hybrid screen with the *S. cerevisiae* genomic library should not yield as many true or false positives. Screening the *S. cerevisiae* library with the Ad2 E1B 55-kDa protein is appropriate because we know that it is biologically active in yeast. Induced expression of the E1B protein fused to a NLS in *S. cerevisiae* cells inhibits cell growth (Liang et al., 1995). NLS-E1B is localized to the yeast nucleus and leads to accumulation of nuclear poly(A)+ RNA. It is expected that screening the *S cerevisiae* genomic library will reveal possible human proteins that will bind to E1B 55-kDa. Human proteins containing the same evolutionarily conserved domains of the yeast proteins or functional human homologues may also interact with E1B 55-kDa. Simultaneously screening both the Hela cDNA library and the *S. cerevisiae* genomic library increases the opportunity of finding human E1B 55-kDa interacting proteins.

We also expect that screening expression libraries with an E1B 55-kDa fragment of human Ad2 may further our understanding of the effects of E1B 55-kDa of

highly oncogenic Ad12. Proteins positive for Ad2 E1B 55-kDa binding would also be tested for binding to Ad12 E1B 55-kDa, which proved too toxic for the host yeast to be used as bait. Expressing Ad12 E1B 55-kDa had a detrimental effect on yeast doubling time. Most of the knowledge that has accumulated on the large E1B protein has come from studies with non-oncogenic Ad2 or Ad5 of the same subgroup. There is strong sequence similarity between the E1B 55-kDa proteins of serotypes 12 and 2/5, with the greatest differences in the N-terminal third. Downregulation of MCH class 1 by E1A proteins of Ad12 but not Ad2 or Ad5 is believed to play a role in Ad12 oncogenic potential in rodents (Pereira et al., 1995; Schouten et al., 1995).

Introduction to Some E1B-binding Proteins Identified

Screening the Hela cDNA library and the *S. cerevisiae* genomic library identified several E1B 55-kDa-interacting proteins. These include proteins involved in the ubiquitin (Ub) fusion degradation pathway, a major pathway for selective protein degradation in eukaryotes, such as Ub and UbcH7 from the human library and yeast Ufd1. Post-translational conjugation to Ub marks a protein for degradation by the proteasome. UbcH7 is a Ub-conjugating enzyme responsible for Ub-mediated degradation of p53 (Ciechanover, et al., 1994) and c-fos (Stancovski et al., 1995) and processing of the NfκB (nuclear factor-κB) precursor p105 (Orian et al., 1995). Ufd1 is involved at a post-ubiquitination step of the Ub-proteasome pathway and is highly conserved from yeast to humans (Johnson, et al., 1995; Novelli et al., 1998). SUMO-1

[also called UBL1 (Shen et al., 1996), PIC1 (Boddy et al., 1996), GMP1 (Matunis et al., 1996) or sentrin (Okura et al., 1996)] was also found as a result of Hela cDNA library screening to interact with Ad2 E1B 55-kDa. SUMO-1 is a Ub-like protein which covalently modifies a limited number of proteins in a manner similar to ubiquitination (Kamitani et al., 1997). During the process of writing this work it was discovered by Endter et al. (2001) that Ad5 E1B 55-kDa could be modified by SUMO-1 in vivo.

Other interesting proteins identified include yeast Bdf2, functionally redundant with Bdf1 and homologous to the carboxyl-terminal half of mammalian $TAF_{II}250$, the largest subunit of TFIID (Matangkasombut et al., 2000). Bdf1, Bdf2, and $TAF_{II}250$ each contain two copies of the evolutionarily conserved bromodomain. There is also human Clk1 which phosphorylates SR proteins and may play a role in the altered splicing patterns during the switch from early to late phase of adenovirus replication (Duncan et al., 1997), and transcription repressor DAXX, implicated in Fas-induced apoptosis (Yang et al., 1997; Torii et al., 1999). The discovery that human DAXX binds to Ad2 E1B 55-kDa inspired further investigation into the nature of this interaction. The fact that DAXX is suspected to be a pro-apoptotic protein is of particular interest given the involvement of E1B 55-kDa in cellular transformation.

Introduction to DAXX

DAXX contains no homology to other known proteins. Murine DAXX (mDAXX) was originally identified as binding to the intracellular domain of Fas (also named APO-1 and CD95) in a two-hybrid screen by Yang et al. (1997). They showed that the overexpression of mDAXX and Fas together enhanced Fas-mediated apoptosis through the Jun N-terminal kinase (JNK) pathway. Fas is a member of the tumor necrosis factor (TNF) receptor superfamily and is activated when immune effector cells deliver Fas ligand (FasL) to the receptor. Conflicting stories have emerged regarding the role of DAXX in Fas-induced apoptosis. In 1999, Torii et al. showed that sensitivity to apoptosis induced by Fas was enhanced in cells overexpressing the human homologue of mDAXX. This cellular response was specific to Fas. Apoptosis induced by other TNF-family death receptors was not affected. They reported accelerated activation of caspases but no increase in JNK activity. Surprisingly, DAXX may play an anti-apoptotic role in mouse development (Michaelson et al., 1999). A deficiency in DAXX results in extensive apoptosis and embryonic lethality. A recent report claims that Fas-mediated cell death does not involve DAXX (Villunger et al., 2000). A dominant-interfering mutant of DAXX was used in these experiments although they admitted that higher levels of the mutant might have had an effect on FasL-induced apoptosis.

Torii et al. (1999) also showed that DAXX is in fact a nuclear protein that localizes to structures called PML oncogenic domains (PODs) but does not bind Fas. PODs are essentially accumulations of proteins and these multiprotein complexes

associate with the nuclear matrix (Ascoli and Maul, 1991). There are approximately 10-30 PODs per cell nucleus. In normal cells, PML is concentrated within these structures and is critical to the formation of PODs (Ishov et al., 1999). Apart from PML and DAXX, other proteins found in PODs include: p53 (Fogal et al., 2000), co-activator and histone acetyl transferase, CBP (LaMorte et al., 1998), Sp100 recognized by autoantibodies from patients with primary biliary cirrhosis (reviewed in Sternsdorf et al., 1997), BML, the RecQ helicase missing in Bloom's syndrome (Zhong et al., 1999), and pRb (Alcalay et al., 1998) (illustrated in figure 4). The localization of all other proteins to PODs is dependent on SUMO-1 modification of PML (Zhong et al., 2000a). The function of these nuclear bodies in the cell is still not clear but there is evidence for their involvement in DNA replication, transcriptional activation and apoptosis (reviewed in Hodges et al., 1998). PML is a cell growth and tumor suppressor protein essential for multiple apoptotic signals (Wang et al., 1998). These include IFN, TNF, Fas, and ceramide, normally employed to induce apoptosis upon DNA damage or neoplastic transformation. PML is found fused to the retinoic acid receptor-alpha (RARα) in acute promyelocytic leukemia (APL) due to a distinct chromosomal translocation, t(15;17) (reviewed in Melnick and Licht, 1999). The resulting PML/RARα fusion protein contributes to the pathogenesis of APL. PML/RARα can heterodimerize with PML and protect cells from apoptosis, thus acting in a dominant negative manner (Wang et. al. 1998). Also, due to the presence of the fusion protein, PML can no longer mediate assembly of PML nuclear bodies (Dyck et al., 1994; Weis et al., 1994).

DAXX interacts physically with PML and localizes to PODs in mitogen-activated splenocytes (Zhong et al., 2000b). The sequestering of DAXX in PODs by PML is necessary for its pro-apoptotic function (Torii et al., 1999). A mutant DAXX that could no longer localize to PODs was unable to enhance Fas-induced apoptosis. By sequestering DAXX in PODs, the PML protein also inhibits the repressor function of DAXX (Li et al., 2000a). DAXX possesses strong transcriptional repressor activity (Hollenbach et al., 1999; Torri et al., 1999) and so it is possible that DAXX plays a role in programmed cell death by modulating the transcription of certain genes. This repressor function may result from the recruitment of HDACs by DAXX (Li et al., 2000b). HDAC inhibitor, TSA, blocks DAXX repressor activity. DAXX interacts with transcription factor ETS1 and prevents transactivation of at least two genes regulated by ETS1: MMP1, involved in the process of tumor invasion and metastasis, and Bcl2, which is anti-apoptotic (Iwasaka et al., 1996; Li et al., 2000b).

PODs increase both in size and number following interferon (IFN) treatment of cells and may be sites for antiviral defense mechanisms (Hodges et al., 1998). IFNs are growth regulatory cytokines with antiviral activity (reviewed in Jokik, 1991). IFN treatment also dissociates DAXX from centromeres and results in the association of DAXX with PODs (Everett et al., 1999). Several viruses express proteins that specifically modify PODs, including adenoviruses, herpes simplex virus type 1, cytomegalovirus, and human T-cell leukemia virus. E1B 55-kDa itself localizes to PODs shortly after adenovirus infection, along with E4orf3 (Doucas et al., 1996). PODs then undergo a dramatic morphological change from spherical structures to fibrous-like

tracks. Host factors are recruited, along with E1B 55-kDa, from the PODs to viral inclusion bodies, which are sites of adenovirus DNA replication and late RNA transcription (illustrated in figure 4). E4orf3 remains behind in tracks with PML. Experiments with adenovirus deletion mutants point to E4orf3 as the viral protein responsible for POD reorganization, with E1B 55-kDa contributing to the sequential redistribution of POD proteins. However, immunofluorescence co-localization experiments performed here show that E1B 55-kDa itself is capable of disrupting the interaction between DAXX and PML, as well as the formation of PODs. These results suggest that another mechanism by which E1B contributes to cellular transformation may be by preventing the apoptotic response triggered by DAXX following adenovirus infection.

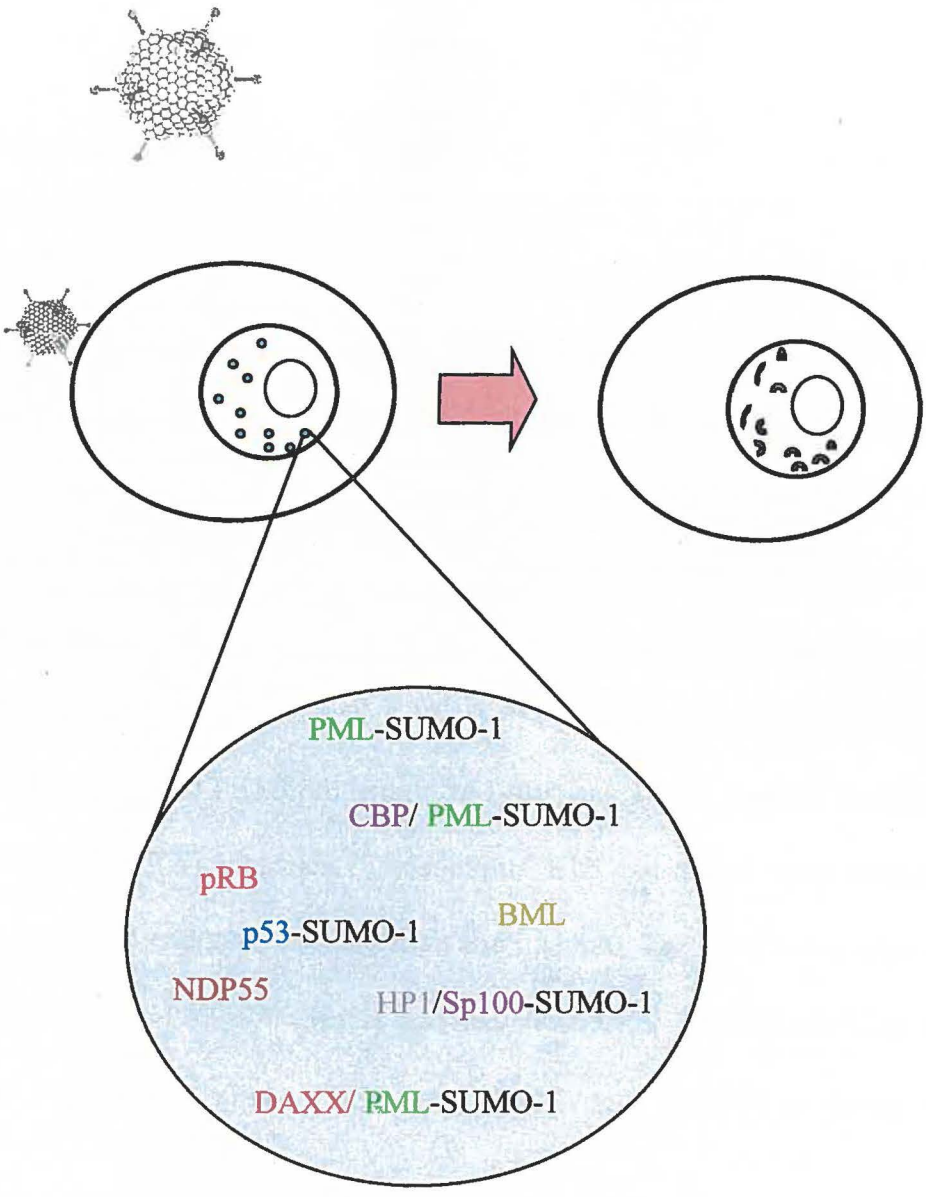

Figure 4: Adenovirus infection leads to disruption of PODs.
Shortly after adenovirus infection E1B 55-kDa and E4orf3 associate with PODs, which undergo a dramatic morphological change into fibrous-like tracks, illustrated here. Host factors are recruited from the PODs to viral inclusion bodies. A single POD and known protein components is also illustrated.

Materials and Methods

Yeast Two-Hybrid Assays

Yeast two-hybrid assays were performed with Ad2 E1B 55-kDa (aa 155-495) fused to the Gal4 BD in plasmid pGBDU-C1 along with either *Saccharomyces cerevisiae* genomic library fragments in plasmid pGAD-C1, or human Hela cDNA library fragments in plasmid pGAD GH, fused to the Gal4 AD (pre-made MATCHMAKER plasmid libraries, CLONTECH Laboratories, Inc.). Two-hybrid assays were also performed with the carboxyl-terminal of DAXX (aa 621-740) fused to the Gal4 AD, isolated from library screening (described below) and several Ad E1B 55-kDa fragments (Ad2 E1B aa 1-437, 155-495, and 437-495; Ad12 E1B full length, aa 1-408, and 1-204) fused to the Gal4 BD, cloned into pGBDU-C(X) plasmids. Adenovirus E1B constructs were cloned into plasmids by restriction endonuclease digestion and ligation. The yeast strain used for all two-hybrid assays was PJ69-4A (*MAT*a *trp1-190 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*) outlined in James et al. (1996).

Co-transformation of plasmids in yeast was done by the basic lithium acetate (LiOAc) method. Several host yeast colonies were used to inoculate synthetic dropout (SD) liquid medium lacking lysine (lys^-). Cultures were grown overnight at 30^0C to an OD_{600} between 0.8 and 1.0, centrifuged at 2,600 rpm for 5 min at RT then washed 2x with sterile H_2O and 1x with filtered LiOAc solution. Pellets were resuspended in 0.5 ml

LiOAc solution for each 50 ml of culture. Next, 0.1 ml of the suspension was added to a 1.5 ml eppendorf containing 10 μg of each plasmid DNA. To test for the presence of contaminants, a control without DNA (H_2O) was performed for each round of transformation. The mixture was left at RT for 10 min, then 0.28 ml of 50% filtered PEG/LiAc solution was added. The tubes were placed at 30°C for 1hr. 43 μl DMSO was added and the samples were heat shocked at 42°C for 5 min. After washing 3x with sterile H_2O, the pellet was resuspended in 1 ml sterile H_2O. 2 volumes of the suspension (0.15 ml and 0.3 ml) were plated onto SD $lys^-leu^-ura^-$ to select for both the pGAD plasmid which contains the gene for leucine (*leu2*) and the pGBDU plasmid which contains the gene for uracil (*ura3*) and incubated 72 hr at 30°C. Where water was used in place of plasmid DNA, no colonies should appear after three days incubation. A single yeast colony was picked from each plate and streaked again onto SD $lys^-leu^-ura^-$. Plates were incubated at 30°C for 48 hrs after which single colonies could be picked and streaked on different SD plates. Protein-protein interactions were recognized by: *1.* growth on SD his^- + agar in the presence of 5 mM 3-amino-1,2,4-triazole (3-AT) to inhibit *his3* reporter gene auto-activation, *2.* growth on SD ade^-, and *3.* detection of β-galactosidase activity in the presence of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside).

To perform the β-galactosidase assays, single colonies (triplicate for each co-transformation) were spread in a straight line on SD $lys^-leu^-ura^-$ + agar and placed at 30°C overnight. A mixture of 5 ml 1% agarose in H_2O, 5 ml heated sodium phosphate buffer, 0.1 ml 10% SDS and 0.2 ml X-Gal was poured over each plate. Following agarose

solidification plates were incubated at 37^0C for 1 hr. By this time, enzyme-producing yeast colonies appear blue. The interaction between Ad2 E1B 55-kDa (aa 155-495) in pGBDU and full length p53 in pGAD was used as a positive control in β-galactosidase assays as well as growth on selective media. Negative controls consisted of replacing one of the co-transformants with empty pGAD or with empty pGBDU.

Library Screening

Figure 5 outlines the steps involved in the identification of Ad2 E1B 55-kDa-interacting proteins. Ad2 E1B 55-kDa (aa 155-495) in pGBDU-C1 (bait) and expression library fragments in pGAD-C1 or pGAD GH (prey) were co-transformed into yeast as described above. Between 5,000 and 8,000 colonies were obtained for each plate. Yeast colonies possessing both plasmids of interest were replica plated onto SD his^- + 5 mM 3-AT and SD ade^-. Those colonies that grew on both plates were picked and grown on SD $lys^-leu^-ura^-ade^-$ to select for protein-protein interaction and ensure plasmid co-segregation. Colonies were then grown on SD leu^- + agar with the addition of 5-fluoroorotic acid (5-FOA) (selects for the loss of the pGBDU/Ad2 E1B plasmid). These colonies were replica plated onto SD ade^- plates. Colonies that grew on SD ade^- + agar in the absence of Ad2 E1B 55-kDa were considered false positives and discarded. The majority of colonies were lost at this step. Recovery of potential positives consisted of isolation of plasmid DNA from yeast and amplification in *E. coli*.

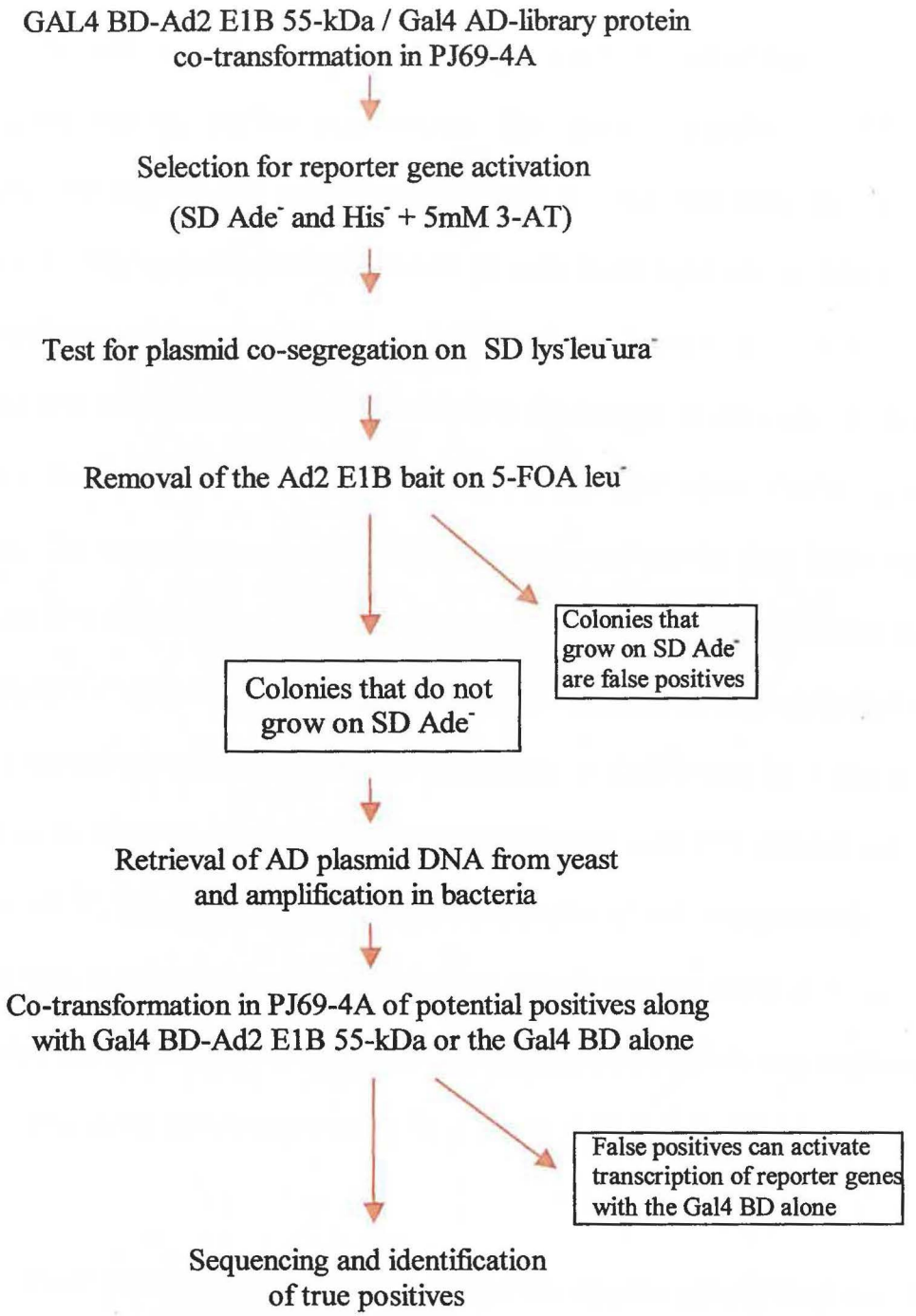

Figure 5: Method used to screen for Ad2 E1B 55-kDa-interacting proteins.
The *S. cerevisiae* genomic library and the Hela cDNA library were screened with Ad2 E1B 55-kDa as bait. Several steps are involved in the elimination of false positives. True positives are proteins, encoded by expression library DNA fragments, which can activate expression of reporter genes only in the presence of the bait.

In order to extract plasmid DNA from yeast, 0.1 ml of liquid SD lys$^-$leu$^-$ was inoculated with one positive yeast colony. The culture was grown at 30^{0}C to an OD_{600} between 0.8 and 1.0 and centrifuged for 5 min at 2,600 rpm (here the pellet could be frozen for later use). Cells were washed 2x with sterile H_2O and 1x with LETS buffer. The pellet was resuspended in 0.3 ml LETS and transferred to a 15 ml tube. 0.3 ml of phenol was added followed by glass beads to the surface of the liquid (1 eppendorf of beads). Next, the tube was vortexed 6x 30 sec at maximum speed alternating with 30 sec on ice. The supernatant was transferred to an eppendorf and the glass beads were washed 1x with 0.15 ml LETS and 1x with 0.15 ml chloroform, collecting the liquid each time in the eppendorf. One volume of phenol/chloroform was added to the eppendorf which was then vortexed for several seconds and centrifuged at 12,000 rpm for 5 min at RT. The DNA in the upper phase was removed and precipitated with 95% ethanol and salt for 10 min at –20^{0}C. The pellet was washed with 95% ethanol and resuspended in 0.3 ml H_2O. 5 µl RNase A (10 mg/ml) was added and the sample was incubated at 37^{0}C for 10 min. A second phenol/chloroform extraction and ethanol precipitation was performed at this step and the pellet was resuspended in 20 µl sterile H_2O.

Yeast DNA (3 µl) was analyzed on a 0.8% agarose gel and later amplified in *E. coli* (XL1 Blue) by electroporation. 1-5 µl of DNA was mixed with 40 µl of competent cells and electroporated at 1.8 kV (Bio-Rad Gene Pulser, 25 µF with the pulse controller set to 200 ohms). Following electroporation, 0.2 ml of YT was added to the cuvette and the cells were plated on YT + agar with 100 µg/ml ampicilin (Amp). Plates were

incubated at 37^{0}C overnight. A single colony was used to inoculate 2 ml YT + Amp. After shaking overnight at 37^{0}C, 0.1 ml of this culture was used to inoculate 250 ml YT + Amp and again grown overnight at 37^{0}C. Bacterial cells were harvested by centrifugation at 6,000 rpm for 15 min at 4^{0}C and resuspended completely in 10 ml solution I. The cell suspension was transferred to a 50 ml centrifuge tube. 10 ml solution II was added and the tube was inverted 4-6 times to mix and incubated at RT for 5 min. 10 ml cold solution III was added to the lysate and mixed by inversion followed by 30 min incubation on ice. After 30 min centrifugation at 15,000 rpm at 4^{0}C, the supernatant was divided into two clean 50 ml centrifuge tubes and each was mixed with 11 ml isopropanol. Tubes were centrifuged immediately at 11,000 rpm at 4^{0}C for 30 min. The DNA pellet was washed with 5 ml 70% ethanol, air-dried and resuspended in 0.5 ml distilled H_2O by vortexing. The DNA solution was transferred to an eppendorf. 5 µl RNase A (10 mg/ml) was added and the sample was incubated at 37^{0}C for 1 hr. Extraction with 0.5 ml phenol consisted of vortexing for several seconds, centrifugation at full speed for 5 min and transferring the upper phase to a clean eppendorf. The extraction was repeated with 0.5 ml phenol/chloroform. 50 µl 3 M NaOAc (pH 5.3) and 0.42 ml isopropanol were added to precipitate the DNA followed by centrifugation at full speed for 10 min. The pellet was washed 1x with 0.25 ml 70% ethanol and 1x with 0.25 ml 95% ethanol, dried and resuspended in 0.2 ml TE. The DNA concentration was determined by measuring the OD_{260} (1 OD_{260} = 50 µg DNA/ml).

pGAD vectors containing the fragments of interest were then co-transformed back into yeast along with either Ad2 E1B 55-kDa in pGBDU-C1 or the pGBDU-C1 plasmid

alone. Those library proteins possessing intrinsic DNA-binding ability or those that could activate transcription of reporter genes, *ade2*, *his3*, and *lacZ*, through association with the Gal4 BD alone were eliminated at this step. Only those positive interactions dependent on the presence of Ad2 E1B 55-kDa were considered true. True positives were sequenced using the DNA sequencing procedure originally described by Sanger and Coulson (1975). The primer used for sequencing was 5'-gatgatgaagatacccc-3', present in the Gal4 AD. The proteins were identified via an on-line BLAST search at the National Center for Biotechnology Information web site.

Protein Expression and Purification

The coding regions of UbcH7wt and UbcH7ΔE2 were generated by PCR, tagged with FLAG at the 5' end and 6X His at the 3' end, and cloned into pET22b(+). The proteins were expressed in *E. coli* BL21(DE3) upon IPTG induction. To do this, a single colony containing the plasmid of interest was picked to inoculate 100 ml YT + Amp and grown overnight in a 37^{0}C shaker. This culture was diluted 1:10 (25 ml in 250 ml YT + Amp) and incubated at 30^{0}C until the OD_{600} was 0.6. IPTG was added to a final concentration of 0.4 mM and flasks were placed at 30^{0}C for 4 hrs. The proteins were then purified using nickel-nitrilotriacetic acid (Ni-NTA) agarose. Cells were harvested by centrifugation at 8,000 rpm at 4^{0}C for 10 min. The pellet was resuspended in 12 ml ice-cold His binding buffer and transferred to two 50 ml tubes. Each was sonicated (at power setting 10 on the sonicator) 10x 20 seconds, centrifuged at 16,000 rpm for 10 min

at 4^0C, and filtered. Resin was prepared by adding 10 ml His binding buffer to 2 ml Ni-NTA agarose. After 3 min centrifugation at 1,200 rpm, 1 ml of resin remained. 10 ml His binding buffer was added and this was placed at 4^0C for 2 hrs, rotating. The resin was spun down and washed 4x with 10 ml His washing buffer before eluting with 5x 1 ml His elution buffer. 10 µl of each elution was examined on a 12% SDS PAGE gel run at 150 volts in electrophoresis buffer. The gel was stained with Coomassie Brilliant blue for 10 min at RT and incubated overnight in de-stain solution. Eluted proteins were dialyzed by transferring the liquid into dialysis tubing secured with clamps. These were placed in dialysis buffer, stirring, overnight at 4^0C. The protein concentrations were determined by preparing three dilutions of bovine serum abumin (BSA) as a protein standard. Standard and sample solutions in 0.8 ml H_2O were added to 0.2 ml of Bio-Rad dye reagent, vortexed, and the absorbance measured at 595 nm. A standard curve was plotted for BSA and used to determine sample concentrations. Ad2 E1B 55-kDa was expressed in insect cells and affinity purified with Ni-NTA agarose.

Immunoprecipitation

UbcH7ΔE2 (200 ng) purified from *E. coli* was incubated with monoclonal antibody, 2A6, against Ad2 E1B 55-kDa (0.15 ml of hybridoma supernatant), or with both 2A6 and Ad2 E1B 55-kDa (100 ng) for 1 hr at 4^0C, rotating. 15 µl of protein G-agarose beads was added and incubated for 1 hr at 4^0C, rotating. Following centrifugation at full speed for 2 min at 4^0C, the beads were washed with 1 ml of wash

buffer 1. This step was repeated with wash buffer 2 and wash buffer 3. Beads were then resuspended in 20 µl SDS loading buffer and placed at 100^0C for three min. The precipitates were separated on a 12% gel by SDS-PAGE and the proteins were transferred to a nitrocellulose membrane (Ply Screen PVDF transfer membrane) for 2 hrs at 150 volts at 4^0C in transfer buffer to be analyzed by Western blot. The membrane was blocked for 1hr at RT with 5% milk in Tris-buffered saline (TBS) and 0.1% Tween 20 and probed with monoclonal antibody against the FLAG epitope (1:1000 dilution in blocking buffer) for 1 hr at RT. Following washes with TBS-Tween 20 (3x 10 min at RT), the membrane was incubated with secondary antibody, goat anti-mouse for 1hr (1:1000 dilution in blocking buffer). The membrane was treated with enhanced luminol reagent (NEN Life Science Technologies) for 60 sec and exposed to film.

Cell Culture

Three different cell lines were cultured: G401, a rhabdoid kidney tumor cell line, G401 CC3, a derivative of G4O1 expressing Ad12 E1B 55-kDa protein and 293, human embryonic kidney cells expressing Ad5 E1A and E1B proteins. 293, G401, G401 CC3, cells were grown in DMEM medium supplemented with 10% fetal bovine serum. Added to the G401 and G401 CC3 media were 15 µg/ml hypoxanthine, and 10 µg/ml thymidine. G401 CC3 medium also contained 250 µg/ml G418. Cells were incubated in a humidified chamber at 37^0C with a 5% CO_2 environment.

Immunofluorescence Microscopy

The localization patterns of PML and DAXX were examined in three different cell lines: G401, G401 CC3, and 293. Cells were grown overnight on a glass coverslip to approximately 70% confluence, fixed with 4% paraformaldehyde for 20 min at room temperature (RT) and permeabilized with 0.2% Triton X in PBS for 15 min at RT. After blocking for one hour in blocking buffer (2% fetal bovine serum, 0.1% sodium azide, 0.1% Tween 20 in PBS) the cells were incubated with both a rabbit anti-DAXX antibody (1:500 dilution in blocking buffer) and a mouse anti-PML antibody (1:250 dilution in blocking buffer) for 2 hrs. The cells were washed 3x with TBS and then incubated for 1hr with a mixture of fluorochrome-labeled secondary antibodies: goat anti-rabbit conjugated to Texas red and FITC-conjugated goat anti-mouse (each diluted 1:100 in blocking buffer). The cells were washed again 3x with TBS followed by staining for 10 min with 4',6'-diamidino-2-phenylindole (DAPI) (0.2 μg/ml in PBS) at RT. Localization of both DAXX and Ad12 E1B 55-kDa was examined in the G401 CC3 cell line. Cells were prepared as above with primary antibodies consisting of mouse anti-DAXX antibody (1:500 dilution in blocking buffer), and rabbit alpha-E1B (1:1000 dilution in blocking buffer). Fluorochrome-labeled secondary antibodies used were Texas red-conjugated goat anti-mouse, and FITC-conjugated goat anti-rabbit (each diluted 1:100 in blocking buffer).

PML cellular localization was also examined in G401, G401 CC3 and 293 cell lines. In place of DAPI, cells were stained with propidium iodide (PI). Cells were

incubated with a mixture of primary antibody, mouse alpha-PML, and RNase A (0.3 mg/ml in PBS) for 2hrs at 37^0C. The secondary antibody used was FITC-conjugated goat anti-mouse. After washing 3x with TBS-Tween 20 the cells were stained for 10 min with PI (1.0 μg/ml in PBS) at RT.

In all cases coverslips were mounted and viewed using an Olympus IX-70 scanning laser biological microscope. An Omnichrome ion laser power supply and NEC gas laser power supply were connected to the Olympus Fluoview system. The laser line filter turret was set at 568 and 488 to allow argon and krypton laser combination. The laser intensity was set at 0.6 and the confocal aperture at 0.2. Cells were examined with the 40X objective (LCPlanFI) with oil. A Lambda 10-C optical filter changer was used to choose the appropriate filters. Images were captured using Fluoview software (version 2.1.37) and stored as *tif* files.

Results

Potential Ad2 E1B 55-kDa Cellular Targets

The yeast two-hybrid method was employed for screening the *Saccharomyces cerevisiae* genomic library and the Hela cDNA library to identify proteins that associate with Ad E1B 55-kDa. The first step concerned choosing an E1B construct that was not toxic to cells, did not activate transcription alone, and tested positive in yeast two-hybrid assays against a protein that it was known to interact with. This last step would help to assure that the E1B/Gal4 DNA-binding domain (BD) fusion protein was properly folded. Ad2 E1B 55-kDa (aa 155-495) fused to the Gal4 BD was the bait hybrid used for library screening. It associates with itself and with p53 fused to the Gal4 transcription activation domain (AD) in two-hybrid assays (data not shown). Proteins encoded by pre-made MATCHMAKER plasmid libraries were fused to the Gal4 AD. The insert size range was 0.4 – 2.0 kb with an average insert size of 1.5 kb.

The rationale behind screening a yeast library with the large human adenovirus E1B protein was that human homologues of yeast proteins positive for E1B-binding, or evolutionarily conserved functional domains, could be identified. The complete DNA sequence of the *S. cerevisiae* genome has been determined and made available on the web. Alternatively, the biological effects of interactions could be studied in yeast where many cellular processes are less complex. Partial screening of the *S. cerevisiae* genomic library (10^6 transformants) has revealed 8 different yeast proteins positive for Ad2 E1B

55-kDa binding. These proteins are listed in table 3 along with their functions in yeast and comparison to human sequences.

Potential *S. cerevisiae* Ad2 E1B 55-kDa-interacting proteins include UFD1, for ubiquitin (Ub) fusion degradation, a 40-kDa protein essential for cell survival in yeast (Johnson et al., 1995). UFD1 has not been fully characterized but it is implicated at a post-ubiquitination step of the Ub-fusion degradation pathway. UFD1 is also found complexed to Np14, implicated in nuclear transport (Meyer et al., 2000). A human homologue of UFD1, called UFD1L, has been described as a potential housekeeping gene (Novelli et al., 1998). UFD1L haploinsufficiency contributes to the phenotype seen in 22q11 deletion syndrome (Yamagishi et al., 1999). Next is RIS1 (also known as DIS1). RIS1 interferes with silencing and may act by increasing accessibility of silenced DNA. It is a member of the SWI/SNF2 family of DNA-dependent ATPases (Zhang and Buchman, 1997) and shares sequence homology with a number of human proteins including DNA-dependent ATPases, helicases, repair protein ERCC6, and centromere protein, CENP-F. The region covered by both RIS1 cDNA fragments identified by library screening shows CENP-F homology. CENP-F is a cell cycle specific nuclear autoantigen that associates with both the nuclear matrix and centromeres (Liao et al., 1995). To determine whether it binds to Ad2 E1B 55-kDa, CENP-F was cloned into the pGAD plasmid. Gal4 AD/CENP-F interacted with the Gal4 BD/Ad2 E1B 55-kDa in yeast two-hybrid assays but not with Gal4 BD alone (data not shown). Next is Bdf2, functionally redundant with Bdf1 and homologous to the carboxyl-terminal half of mammalian $TAF_{II}250$, the largest subunit of TFIID (Matangkasombut et al., 2000).

Bdf1, Bdf2, and $TAF_{II}250$ each contain two copies of the bromodomain, a 110 amino acid module conserved from yeast to human (Jeanmougin et al., 1997). $TAF_{II}250$ may consist of two separate proteins in yeast, Bdf1/Bdf2 and Taf145 which possesses acetyltransferase activity. The bromodomain is found in other histone acetyltransferases (HATs) including P/CAF, p300/CBP and in the ATPase subunit of SWI/SNF and their homologues. Despite widespread conservation the functional significance of the bromodomain remains unknown although evidence suggests that it may play a role in chromatin remodeling. It was demonstrated that the bromodomain has specificity for acetyl-lysine (Dhalluin et al., 1999) and interacts with histones H3 and H4 (Owen et al., 2000 and references therein). A fragment of Bdf1 that includes one of the two bromodomains has binding affinity for H3 and H4 (Pamblanco et al., 2001). Also, a recent study indicates that the bromodomain of p300 mediates stable association with chromatin (Manning et al., 2001). Next is Fob1, shown to be required for recombination hot spot activity and replication fork blocking within ribosomal RNA genes (rDNA) (Kobayashi and Horiuchi, 1996; Kobayashi et al., 1998). This block has been linked to the generation of circular species of rDNA and aging in yeast (Defossez et al., 1999). Next is FIR1. Relatively little is known about FIR1 except that it interacts with REF2 in yeast two-hybrid assays (Russnak et al., 1996). REF2 is responsible for the 3' end cleavage of yeast mRNA prior to the addition of a poly-A tail. Another hit resulting from screening the yeast library was KRI1. KRI1 interacts with KRR1 (containing a KRR motif) to form a complex that is required for formation of 40S ribosome subunits in the nucleolus (Sasaki et al., 2000). Both proteins are conserved among eukaryotes. Two Yap transcription factors, 33-kDa Yap4 (also known as CIN5) and 44-kDa Yap6, were also

found to bind Ad2 E1B 55-kDa in yeast two-hybrid assays. Yap proteins are AP-1 factors with distinct DNA-binding specificity (Fernandes et al., 1997). There are a total of eight Yap family members defined by particular residues within the basic region of the bZIP domain. Yap4 differs from all other Yap proteins in that Yap4 mutations increase chromosome instability. Unknown open reading frames not listed in table 3 are: YHR134w, YHR073w, YPL138C, and YPL277C.

Table 3: *S. cerevisiae* proteins positive for interaction with Ad2 E1B 55-kDa

Identity	Size and Span of Fragment	Chromosome Location	Protein Size (kDa)	Protein Location	Comments	Compared with **Human** Protein Sequences
Ufd1	700 bp begins 438 bp 3' of the ORF start (ORF=1086 bp)	7	40	cytoplasm	Acts at a post-ubiquitination step of the ubiquitin-fusion degradation pathway	Human Ufd1L is missing in 22q11 deletion syndrome
RIS1	4 kb and 900 bp both begin 1005 bp 3' of the ORF start (ORF=4860 bp)	15	184	nucleus	Previously DIS1 - involved in silencing	Homology to human ERCC6, CENP-F, DNA-dependent ATPases, helicases and hsnf2h
Bdf2	2.7 kb begins 576 bp 3' of the ORF, 3 kb begins 162 bp 3' of the ORF start (ORF=1917 bp)	4	72	nucleus	Unknown function - contains 2 copies of the bromodomain	Homologous to the carboxyl-terminal half of mammalian TAFII250
Fob1	2.2 kb begins 24 bp 3' of the ORF start (ORF=1701 bp)	4	65	nucleolus	Replication fork blocking protein	Replication fork blocking sites have been identified in humans
FIR1	1 kb begins 2346 bp 3' of the ORF start (ORF= 2778 bp)	5	105	?	Also known as PIP1 - unknown function Interacts with REF2	REF2 is involved in 3' end polyadenylation
KRI1	2.5 kb and 5 kb both begin 555 bp 3' of the ORF start (ORF= 1777 bp)	14	69	nucleolus	Unknown function - interacts with KRR1	KRR1 is homologous to human EST of unknown function
Yap4	1.3kb begins 109 bp 3' of the ORF start (ORF=888 bp)	15	33	nucleus	Also known as cin5 - leucine zipper transcription factor	Yaps were first named as yeast AP-1 factors
Yap6	2.5 kb begins 399 bp 3' of the ORF start (ORF=1152 bp)	4	44	nucleus	Leucine zipper transcription factor	Yaps were first named as yeast AP-1 factors

Human proteins positive for Ad2 E1B 55-kDa binding were determined through partial screening of the Hela cDNA library (700 000 transformants). A total of 11 different proteins have been identified. These are listed, along with their functions and other features, in table 4. Excluded from tables 3 and 4 are 'nonsense' peptides, cloned out of frame or in the reverse orientation. As a result of Hela cDNA library screening Ub was found to bind Ad2 E1B 55-kDa. Ub is a highly conserved protein consisting of 76 amino acid residues. Covalent ligation to Ub targets abnormal or short-lived proteins for degradation by the 26S proteasome. The minimum signal for efficient proteasomal targeting is a tetra-Ub chain (Thrower et al., 2000). There are three classes of enzymes involved in ubiquitination: the E1, or Ub-activating enzyme, leads to activated Ub which is covalently linked to E1 via a thioester bond. Activated Ub is transferred to the E2, or Ub-conjugating enzyme, which may donate Ub directly to proteins or may require an E3, or Ub-protein ligase, thought to play a role in substrate recognition. The human Ub-conjugating enzyme, UbcH7 (also called E2F1) was picked up from library screening. UbcH7 is a 17-kDa protein responsible for Ub-mediated degradation of the tumor suppressor protein, p53 (Ciechanover, et al., 1994) and oncoprotein c-fos (Stancovski et al., 1995). Proteolysis of p53 and c-fos by this degradation pathway allows for rapid changes in protein level. UbcH7 is also responsible for processing of the NfκB precursor p105 (Orian et al., 1995). p105 is degraded to the p50 subunit of the transcription factor NfκB which targets genes involved in cell growth and differentiation, inflammation, lymphocyte activation, and the acute-phase response. SUMO-1, an acronym for small Ub-like modifier, was also found to bind to Ad2 E1B 55-kDa as a result of library screening. SUMO-1 covalently modifies a limited number of proteins in a manner

similar to ubiquitination, referred to as sumolation (Kamitani et al., 1997). It consists of 101 amino acid residues, 18% identical and 48% similar to Ub. Unlike Ub, however, the post-translational conjugation of SUMO-1 to various proteins does not target them for degradation. A growing list of cellular proteins known to be modified by SUMO-1 includes p53 (Gostissa et al., 1999), RanGAP1 (Matunis et al., 1996), and PML (Boddy et al., 1996). The conjugation of SUMO-1 to p53 has been reported to result in an increase in p53 transactivation activity by some (Gostissa et al., 1999; Rodriguez et al., 1999) but contradicted recently by Kwek et al. (2001). Sumolation localizes RanGAP1 to the nuclear pore complex (NPC) (Matunis et al., 1996). RanGAP1 is a regulator of the Ran GTP/GDP cycle and therefore the bi-directional transport of proteins and ribonucleoproteins across the NPC (Melchior et al., 1993; Moore and Blobel, 1993). Three molecules of SUMO-1 are conjugated to PML at three separate sites (Kamitani et al., 1998) whereas only one molecule is conjugated to other SUMO-1 protein targets. Sumolation of PML is a prerequisite for POD (PML oncogenic domain) formation, affecting the localization of PML and thus all other POD components to the nuclear structures (Zhong et al., 2000a). Adenoviruses specifically modify PODs, with E1B 55-kDa itself localizing to PODs shortly after infection (Doucas et al., 1996). During the preparation of this manuscript it was reported by Endter et al. (2001) that Ad5 E1B 55-kDa could be modified by SUMO-1 in vivo at a single lysine residue (K104). Sumolation affects E1B subcellular localization and is required for inhibition of p53 transactivation function and transformation by Ad5 E1B 55-kDa. Next in the list of potential E1B 55-kDa binding partners is DAXX, a transcription repressor implicated in Fas-induced

apoptosis (Torii et al., 1999; Yang et al., 1997). DAXX interacts with SUMO-1-modified PML and localizes to PODs (Torii et al., 1999).

Aside from Ub, UbcH7, SUMO-1, and DAXX, other potential human Ad2 E1B 55-kDa-interacting proteins include CLK1, which phosphorylates serine/arginine-rich (SR) proteins. SR proteins are essential splicing factors (Cáceres et al., 1994; Fu, 1993) thought to be regulated by phosphorylation (Xu and Manley, 1997). CLK1 may play a role in the alternative splicing patterns during the switch from early to late phase of adenovirus replication. Overexpression of CLK1 kinase shifted the splicing of E1A pre-mRNA from multiple mRNA products, 9S, 12S and 13S, to the 9S RNA isoform characteristic of the late phase of infection by adenovirus (Duncan et al., 1997). Next is MCM2, required for initiation of DNA replication (Todorov et al., 1994) and implicated in transcription by RNA polymerase II (Yankulov et al., 1999). MCM2 is a mini-chromosome maintenance protein, of which there are at least six (MCM2-7). In all eukaryotes MCM2-7 ensure that DNA replicates only once in each cell cycle (for a review on MCM2-7 see Tye, 1999). During the transition from G1-phase to S-phase MCM2-7 are loaded onto replication origins as part of the pre-replication complex. Phosphorylation of MCM2 alters the conformation of the MCM complex and leads to melting of origin DNA. Next is GG2-1, induced by TNF but of unknown function (Horrevoets et al., 1999). GG2-1 shows no homology at the nucleotide, amino acid, or structural level with any protein or gene present in the combined NCBI and EMBL databases. Another potential E1B partner is NTH1, a functional human homologue of *E. coli* endonuclease III (Nth) (Ikeda et al., 1998 and references therein). NTH1 is involved

in pyrimidine base excision repair. Next is γ-Glutamyl hydrolase (GGH). GGH is a ubiquitous enzyme highly sequestered in the lysosome (Waltham et al., 1997). It has both exo- and endopeptidase activity and is implicated in antifolate resistance. Next is PMSCL1, a component of the PM-SCL particle, a multi subunit complex recognized by autoimmune sera of patients suffering from polymyositis/scleroderma overlap syndrome. PM-SCL proteins are thought to function in RNA processing and degradation pathways (Allmang et al., 1999; Briggs et al., 1998). Lastly is metastasis-associated protein, MTA1. MTA1 overexpression correlates with tumor invasiveness and the presence of metastases (Toh et al., 1997). MTA1 may interact with HDAC1 (Toh et al., 2000) although a role for MTA1 in the alteration of chromatin structure has not been uncovered.

Table 4: Human proteins positive for interaction with Ad2 E1B 55-kDa

Identity	Size and Span of Fragment	Chromosome Location	Protein Size (kDa)	Protein Location	Functions
Ubiquitin	2 x 600 bp, both begin 70 bp 3' of the ORF start and 450 bp begins 59 bp 3' of the ORF start (ORF=487 bp)	pseudogenes/ 12q24.3/ 17p11.2-12	8.6	cytoplasm	Can be conjugated to other proteins marking them for degradation by the proteasome
UbcH7	850 bp covers entire ORF (465bp), missing exon 2 of 4	22q11.2	17	cytoplasm	Ubiquitin-conjugating enzyme specific for c-fos, NFkB, p53 and c-myc
SUMO-1	1.1 kb covers entire ORF (305 bp)	2q33	22	cytoplasm	Can be conjugated to other proteins in a manner similar to ubiquitination
DAXX	2 x 600 bp, both begin 1860 bp 3' of the ORF start (ORF=2220 bp)	6p21.3	120	nucleus/ cytoplasm	Stimulates Fas-induced apoptosis
CLK-1	1.1 kb begins 83 bp 5' of the ORF start (alternatively spliced: 5.6, 3.2, 1.8, 1.7 kb)	2q33	57	nucleus/ cytoplasm	Protein kinase which plays a role in alternative splicing
MCM2	300 bp begins 1584 bp 3' of the ORF start (ORF=1631 bp)	3q21	125	nucleus	Minichromosome maintanance protein involved in the initiation of DNA synthesis
GG2-1	1.6 and 1.5 kb cover entire ORF (ORF=567 bp)	5	?	?	Unknown function, TNF-induced
NTH1	900 bp begins 42 bp 3' of the ORF start (ORF=1028 bp)	16p13	36	nucleus	Functional homologue of *E. coli* endonuclease III
GGH	1.3 kb covers entire ORF (704 bp)	8	36	lysosome	Gamma-glutamyl hydrolase, lysosomal peptidase involved in production of folic acid
PMSCL1	1.2 kb begins 233 bp 3' of the ORF start (ORF=1119 bp)	4q27	75	nucleolus	Polyomyositis/scleroderma autoantigen (ORF=1119 bp)
MTA1	1 kb begins 1115 bp 3' of the ORF start (ORF=2148 bp)	14q32.3	80	nucleus	Metastasis-associated protein

Ad2 E1B 55-kDa Interacts With a Form of UbcH7 Missing Exon 2

cDNA sequencing revealed that the Ub-conjugating enzyme, UbcH7, identified by Hela cDNA library screening with Ad2 E1B 55-kDa as bait, is missing exon 2. UbcH7 is normally spliced to a 462 bp product consisting of 4 exons (Moynihan et al., 1998). The UbcH7 found (ΔE2) is 348 bp consisting of exons 1, 3, and 4. This alternatively spliced variant has not been described in the literature. Figure 6 shows an alignment between wild type (wt) UbcH7 and UbcH7ΔE2 picked up from screening. The cDNA obtained in the yeast two-hybrid screen represented the entire open reading frame, with the exception of exon 2, beginning 11 bp N-terminal of the start methionine and including untranslated sequence 3' of the stop codon. The coding regions of UbcH7wt and UbcH7ΔE2 were generated by PCR and tagged with FLAG at the 5' end and 6X His at the 3' end. Recombinant fusion proteins were expressed in *E. coli* and purified with Ni-NTA agarose. Proteins were resolved on a 12% polyacrylamide gel by SDS PAGE and stained with Coomassie Brilliant blue. The apparent molecular mass of tagged UbcH7ΔE2 is approximately 15-kDa (figure 7B), compared to UbcH7wt at approximately 17-kDa (figure 7A). To verify yeast two-hybrid results, the interaction between Ad2 E1B 55-kDa and UbcH7ΔE2 was assayed by immunoprecipitation (figure 7C). FLAG/His tagged UbcH7ΔE2 was incubated overnight with monoclonal antibody 2A6 against Ad2 E1B 55-kDa or with both 2A6 and Ad2 E1B 55-kDa purified from insect cells. In both cases protein sepharose beads were present in the mixture. The Western blot antibody against the FLAG epitope was used to detect UbcH7ΔE2. Precipitation of UbcH7ΔE2 requires

the presence of the E1B protein (figure 7C, lane 2), indicating that the two proteins interact specifically with each other.

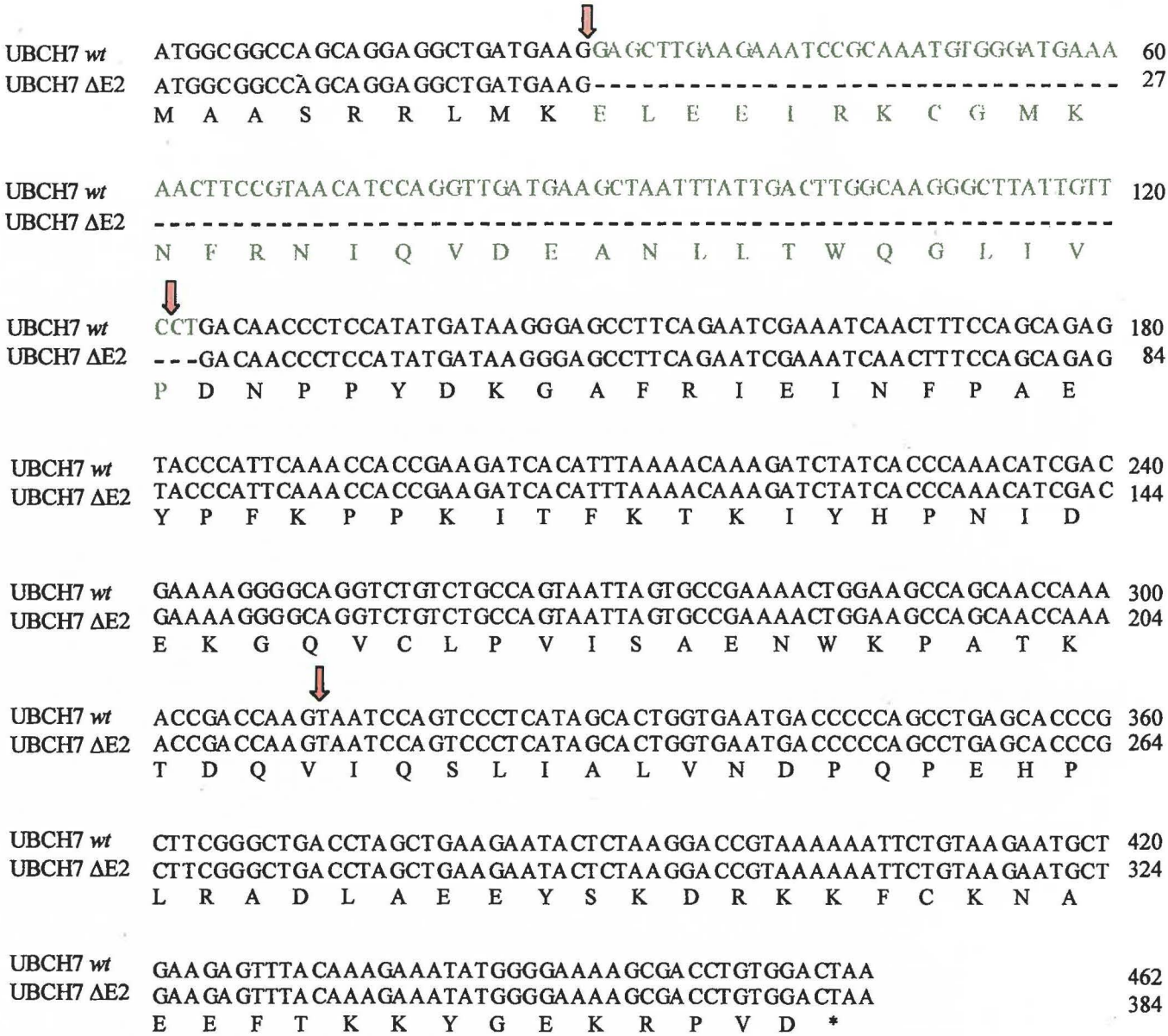

Figure 6: Sequence alignment between wild type UbcH7 and UbcH7 recovered from the Hela cDNA library.

Open reading frame nucleotide sequence and predicted amino acid sequence of UbcH7 wild type (*wt*) and UbcH7 missing exon 2 (ΔE2). UbcH7 (ΔE2) was identified by Hela cDNA library screening as binding to Ad2 E1B 55-kDa. Arrows indicate the positions where the sequence is interrupted by introns. Exon 2 is in green. The (*) denotes the stop codon.

Modified from: Moynihan et al., 1998

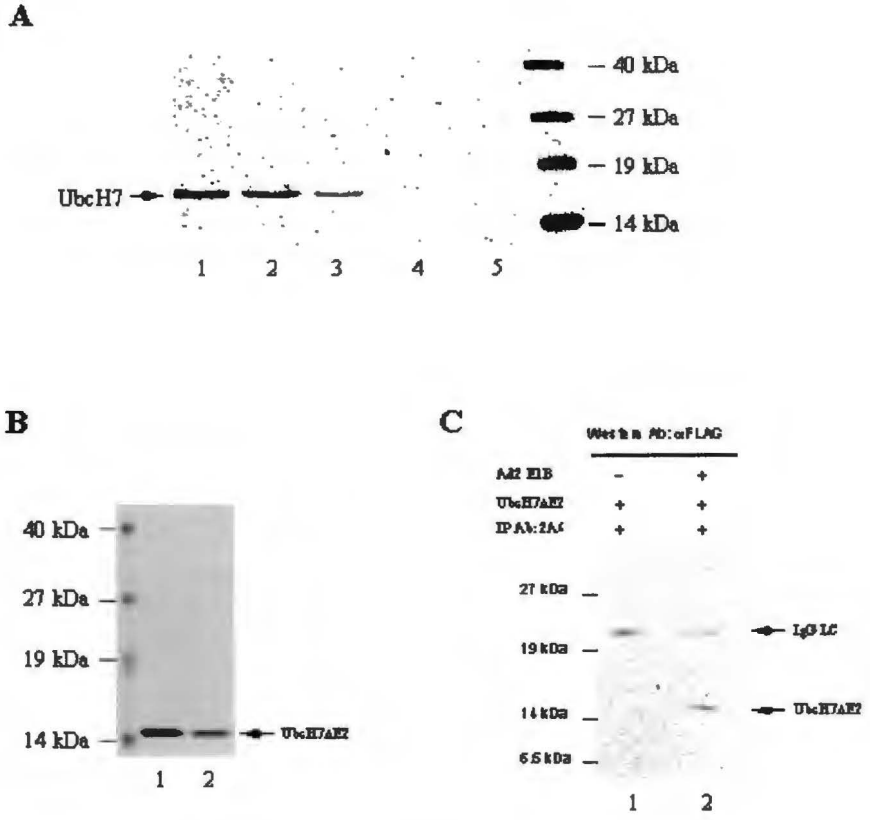

Figure 7: Purification of UbcH7 and UbcH7ΔE2.

The coding regions of UbcH7 and UbcH7ΔE2 were generated by PCR, tagged with FLAG at the 5' end and 6X His at the 3' end, and cloned into pET22b(+). The proteins were expressed in *E. coli* upon IPTG induction and purified using Ni-NTA agarose. The proteins were resolved on 12% gel by SDS-PAGE and stained with Coomassie brilliant blue (A and B). (A) Purification of UbcH7. Different fractions of elution with 200 mM imidazole are shown. (B) Purification of UbcH7ΔE2. (C) Interaction between Ad2 E1B 55-kDa protein and UbcH7ΔE2 assayed using immunoprecipitation. UbcH7ΔE2 purified from *E. coli* and was incubated with monoclonal antibody 2A6 against Ad2 E1B 55-kDa (lane 1), or with both 2A6 and E1B (lane 2). The precipitates were separated with a 12% gel by SDS-PAGE, and the proteins were transferred onto a nitrocellulose membrane and detected with monoclonal antibody against the FLAG epitope. Precipitation of UbcH7ΔE2 requires the presence of E1B (lane 2), indicating that the two proteins interact specifically with each other. IgG LC: immunoglobulin G light chain.

The C-Terminus of Ad E1B 55-kDa Binds to DAXX in Two-Hybrid Assays

DAXX was identified as a potential Ad2 E1B 55-kDa binding partner following Hela cDNA library screening. DAXX is a transcription repressor that promotes sensitivity to Fas. The C-terminal region of DAXX (aa 621-740) was responsible for Ad E1B binding. This domain is required for DAXX enhancement of Fas-induced apoptosis, localization to PODs, and optimal repression of transcription (Torii et al., 1999). The C-terminus of DAXX is also involved in interactions with several cellular proteins including the main component of PODs, PML (Zhong et al., 2000b), ETS1 (Li et al., 2000b), DNA methyltransferase (Michaelson et al., 1999), and CENP-C (Pluta et al., 1998).

The interactions of various Ad E1B 55-kDa deletion mutants fused to the Gal4 BD, with the C-terminus of DAXX (aa 621-740) fused to the Gal4 AD, were assayed using the yeast two-hybrid system. An Ad2 E1B 55-kDa C-terminal deletion (aa 1-437) did not bind to DAXX. No growth was seen on plates lacking histidine (figure 8C) or adenine (not shown). An Ad2 E1B N-terminal deletion (aa 155-495), along with an Ad2 E1B C-terminal fragment (aa 437-495), were positive for DAXX interaction and grew on plates lacking histidine (figure 8C) and adenine (not shown).

Ad2 and Ad12 E1B 55-kDa proteins share a high degree of sequence similarity in the C-terminal region (aa 153-499). For this reason we tested whether Ad12 E1B could also bind to DAXX in two-hybrid assays. DAXX (aa 621-740) interacted with full length

Ad12 E1B but not Ad12 E1B C-terminal deletion mutants (aa 1-408 and 1-204) as seen in figure 8C. Therefore, the C-terminus of DAXX associates with the C-terminus of both Ad2 and Ad12 E1B 55-kDa proteins.

Ad E1B 55-kDa Disrupts POD Formation and PML/DAXX Co-Localization

The pro-apoptotic ability of DAXX is dependent on the physical interaction of DAXX with PML and the consequent localization of DAXX into PODs (Torii et al., 1999). The mechanism by which DAXX induces apoptosis from PODs is yet to be elucidated but given that DAXX is a transcriptional repressor, one possibility is that DAXX represses transcription of genes that promote apoptosis when outside of PODs. Ad E1B 55-kDa localizes to PODs shortly after viral infection (Doucas et al., 1996). With both Ad E1B 55-kDa and PML binding to the same domain of DAXX it is possible that the E1B protein prevents PML from interacting with DAXX and thus prevents the accumulation of DAXX in PODs. To examine the effect of Ad E1B 55-kDa expression on PML/DAXX co-localization, endogenous labeling patterns of PML and DAXX were compared in three cell lines: G401, a rhabdoid kidney tumor cell line, G401 CC3, a derivative of G401 expressing Ad12 E1B 55-kDa protein, and 293, human embryonic kidney cells expressing Ad5 E1A and E1B proteins. Cells were fixed and incubated first with both mouse anti-PML and rabbit anti-DAXX antibodies followed by labeled secondary antibodies: FITC-conjugated goat anti-mouse, and goat anti-rabbit conjugated

to Texas red. Localization of these proteins was analyzed by immunofluorescence microscopy.

The localization of PML and DAXX in G401 cells is seen in figure 9A and 9B respectively. In these cells both proteins are found in discrete areas in the nucleus. There is almost complete co-localization of PML and DAXX in G401 cells as observed by merging the images (figure 9C) where co-localization is seen in yellow. This is characteristic of the localization of PML and DAXX in PODs (Torii et al., 1999). Panels D and E of figure 9 show the localization of PML and DAXX in G401 CC3 cells respectively. Due to the presence of Ad12 E1B 55-kDa both proteins go from a punctate nuclear staining pattern to a diffuse staining pattern. A few areas of concentrated PML localization remain although it was not determined whether or not they correspond to PODs. There is no obvious overlap in PML and DAXX labeling as visualized by the merged image (figure 9F). Likewise in 293 cells, the presence of Ad5 E1B 55-kDa leads to altered PML and DAXX staining patterns, as seen in figure 9G-I. DAXX is dispersed evenly throughout the nucleus while it is difficult to ascertain if PML, although diffused, remains in the nucleus. The number of areas of concentrated PML in these cells is less than in G401 cells. This may be due to the presence of E1A proteins and E1B 19-kDa.

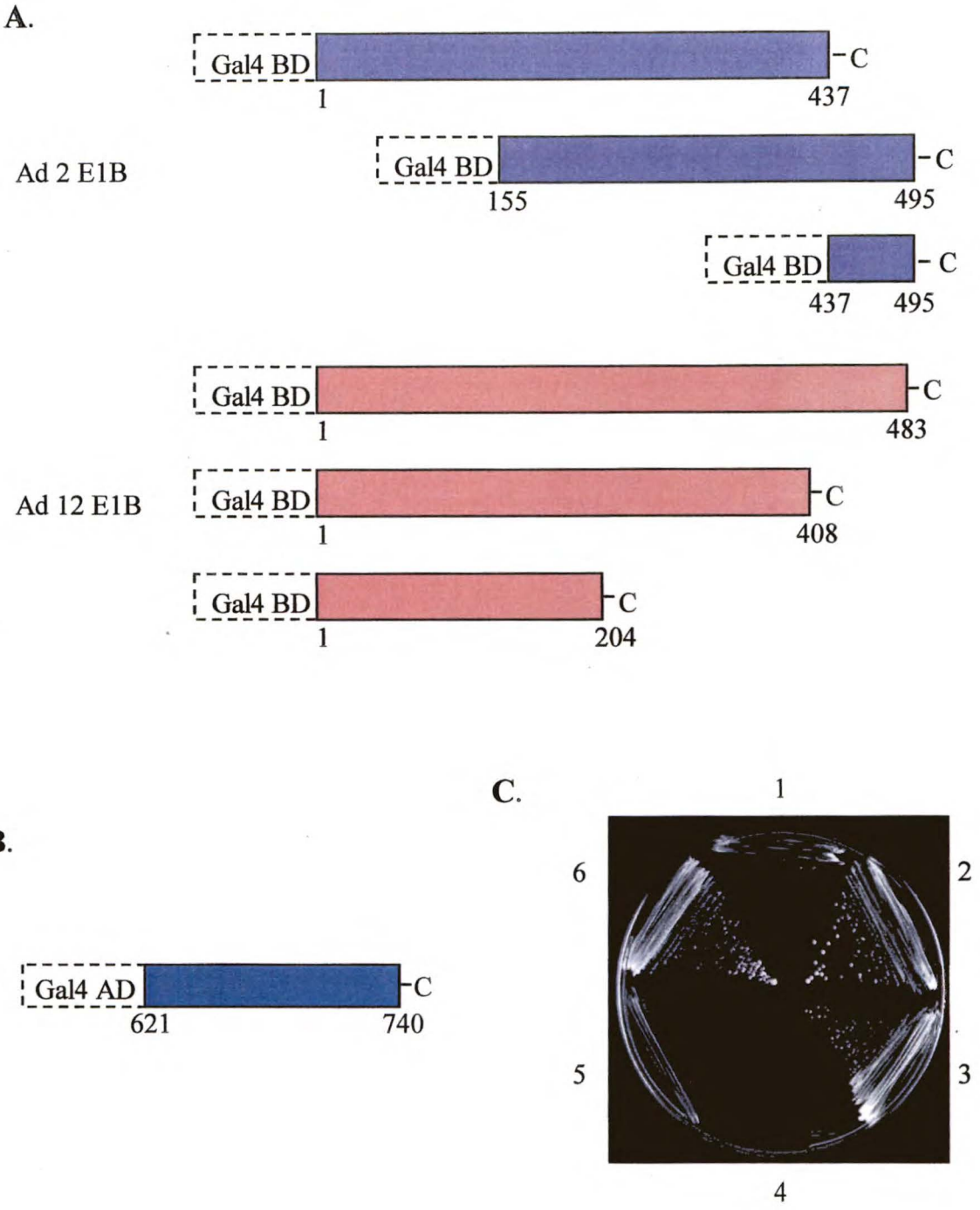

Figure 8: DAXX Interacts with the C-terminus of Ad E1B 55-kDa Proteins.
Results of two-hybrid assays between various Ad E1B 55-kDa constructs and the C-terminus of DAXX. *A.* Representation of Ad2 E1B 55-kDa deletion mutants along with Ad12 E1B full length and C-terminal deletion mutants, all fused to the Gal4 DNA-binding domain (BD). *B.* DAXX C-terminus, identified by Hela cDNA library screening as binding to Ad 2 E1B 55-kDa, fused to the Gal4 activation domain (AD). *C.* Co-transformants: Daxx (aa 621-740) fused to the Gal4 AD along with (1) Ad2 E1B (aa 1-437), (2) Ad2 E1B (aa 155-495), (3) Ad2 E1B (aa 437-495), (4) Ad12 E1B (aa 1-204), (5) Ad12 E1B (aa 1-408), (6) full length Ad12 E1B, each fused to the Gal4 BD, were grown in the absence of histidine but in the presence of 5 mM 3-aminotriazol.

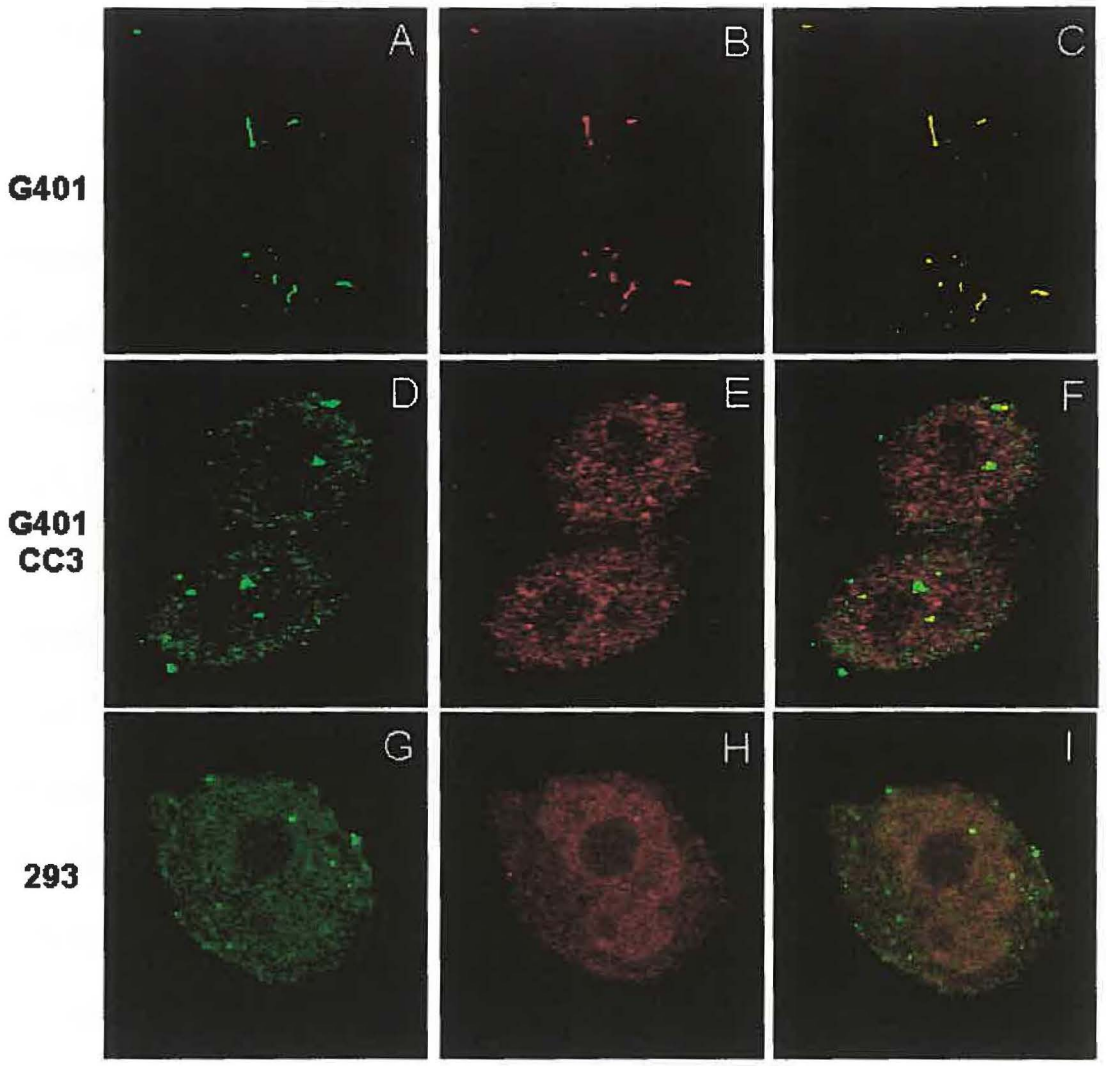

Figure 9: PML and DAXX localization in cells expressing adenovirus E1B 55-kDa proteins.

Immunofluorescence co-localization experiments were performed for PML (*A*, *D*, and *G*) and DAXX (*B*, *E*, and *H*) in three cell lines. *A*, *B*, and *C*: G401, a rhabdoid kidney tumor cell line. *D*, *E*, and *F*: G401 CC3, a derivative of G401 expressing Ad12 E1B 55-kDa protein. *G*,*H*,and *I*: 293 human embryonic kidney cells expressing Ad5 E1A and E1B proteins. Merged images of PML and DAXX: *C*, *F* and *I*.

Ad E1B 55-kDa Affects PML Nuclear Localization

Endogenous PML localization experiments in cells expressing Ad E1B 55-kDa proteins led us to suspect that, apart from the disruption of POD formation, there was an effect on PML nuclear expression. To investigate whether PML was appearing in the cytoplasm, cell nuclei were stained and the location of PML was examined. G401, G401 CC3, and 293 cells were fixed and incubated with mouse anti-PML antibody, followed by labeled secondary antibody FITC-conjugated goat anti-mouse. The cells were then stained with propidium iodide (PI) and as a result, nuclei fluoresce red. As seen in panel A of figure 10, PML is normally concentrated in PODs in G401 cells. Due to the presence of Ad12 or Ad5 E1B 55-kDa proteins (panels B and C of figure 10 respectively) the discrete areas of PML expression are replaced by the diffuse staining pattern. Not only is the nuclear distribution of PML upset, but also the majority of the PML protein is relocalized to the cytoplasm. These single cells are representative of cell populations.

Comparison of Ad12 E1B 55-kDa and DAXX Localization in G401 CC3 Cells

Given the difficulties encountered in immunoprecipitation experiments to verify the DAXX/E1B 55-kDa interactions, protein co-localization was examined in the G401 CC3 cell line by immunofluorescence microscopy. Primary antibodies used were mouse anti-DAXX and rabbit alpha-E1B, along with fluorochrome-labeled secondary antibodies, Texas red-conjugated goat anti-mouse, and FITC-conjugated goat anti-rabbit. Panel A of

figure 11 displays the staining pattern of Ad12 E1B 55-kDa in green. It is distributed throughout the nuclei. DAXX localization in these cells is mainly dispersed (figure 11B) although a few discrete areas of staining can also be detected. With both proteins found throughout the nuclei it is difficult to judge whether or not there is co-localization between Ad12 E1B 55-kDa and DAXX when the two images are merged (figure 11C).

Figure 10: Cellular localization of PML in cells expressing Ad E1B 55-kDa proteins. Cells were stained with propidium iodide and as a result cell nuclei appear red. PML cellular localization was examined using anti-PML primary antibody and FITC-labeled secondary antibody in G401 cells (A), G401 CC3 cells expressing Ad12 E1B 55-kDa (B), and 293 cells expressing Ad5 E1A and E1B proteins (C).

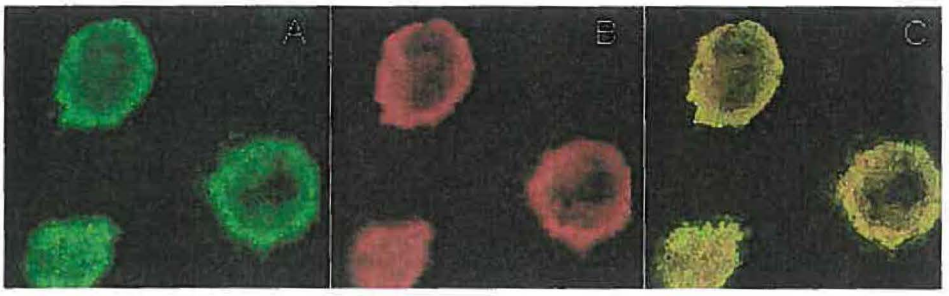

Figure 11: Localization of Ad12 E1B 55-kDa and DAXX in G401 CC3 cells. Immunofluorescence staining of FITC-labeled Ad12 E1B 55-kDa (A), and Texas Red-labeled DAXX (B). C: merged image of panels A and B.

Discussion

The main goal of this research was to identify cellular targets of the Ad E1B 55-kDa protein towards elucidating its function. This was accomplished by yeast two-hybrid screening of expression libraries with Ad2 E1B 55-kDa as bait. An advantage of the yeast two-hybrid method over other protein-protein interaction assays is that detection simply involves the growth of yeast colonies on a plate. Thousands of colonies can be analyzed each round of yeast co-transformation. Partial screening of the expression libraries with Ad2 E1B 55-kDa (a total of 1.7×10^6 transformants) revealed 8 yeast and 11 human proteins positive for E1B-binding (tables 3 and 4 respectively). One of the main limitations of the two-hybrid method is that this technique is known to generate false positives. Extensive testing eliminated proteins that led to reporter gene activation in the absence of the bait (~1500 proteins). The remaining proteins associate specifically with Ad2 E1B 55-kDa in the yeast two-hybrid assays but confirmation of these interactions requires different experimental approaches. Thus, identified interactions must be regarded as putative and be verified by other methods such as immunoprecipitation, cross-linking, etc. A second limitation of the two-hybrid method is that not all potential interacting proteins will be stably expressed as fusion proteins in the nucleus. Due to lack of expression, improper folding of the fusion, or requirements for post-translational modifications, some cellular proteins may be overlooked. There is also a fallback to using a cDNA human library to search for E1B-interacting proteins. Proteins expressed at high levels that bind to the bait may overwhelm the screen resulting in poor representation of proteins that are expressed at low amounts or conditionally

expressed. In fact, it does appear that the human proteins identified as interacting positively with Ad2 E1B 55-kDa in the two-hybrid screen are normally expressed at high levels, although the search was in no way exhaustive. Five of the proteins also came up more than once with 17 'hits' representing 11 different proteins. Beyond these limitations lies the burden of determining the biological significance of each interaction involving the adenovirus bait protein. Protein-interaction studies are useful because they provide a starting point. Complete understanding of protein functionality will call for information on many levels but yeast two-hybrid library screening has pointed towards several prospective avenues of study. Due to time constraints, this project could not begin to verify each E1B-protein interaction or explore the implications of each potential association. The proteins discovered through library screening will be discussed although comments pertaining to the plausibility or possible biological significance of each interaction are speculative.

There are encouraging indicators of the success of the experimental system in identifying cellular targets of Ad E1B 55-kDa. First, the Ub-like protein, SUMO-1, identified as binding specifically to Ad2 E1B 55-kDa, has been reported recently to modify Ad5 E1B 55-kDa in vivo (Endter et al., 2001). Next is the identification of proteins from both yeast and human involved in the Ub-proteasome pathway. These are yeast UFD1, involved at a post-ubiquitination step of the Ub-fusion degradation process, as well as Ub itself from the HeLa cDNA library and human UbcH7, a Ub-conjugating enzyme. One interaction was confirmed in vitro, between Ad2 E1B 55-kDa and UbcH7ΔE2, by immunoprecipitation (figure 7C). The fact that proteins were singled out

more than once from the yeast library, which is constructed from genomic DNA, is also significant. RIS1, Bdf2, and KRI1 were each identified twice. RIS1, a member of the SWI/SNF2 family of DNA-dependent ATPases (Zhang and Buchman, 1997) shares sequence homology with a number of human proteins including DNA-dependent ATPases, helicases, repair protein ERCC6, and centromere protein, CENP-F. Two fragments of RIS1 were picked up during the screen, of 900 bp and 4 kb. Where the two fragments overlap there is homology to CENP-F. This suggested that CENP-F might interact with Ad2 E1B 55-kDa and CENP-F in fact interacted specifically with the E1B bait protein in yeast two-hybrid assays (data not shown). In light of this result it is clear that screening a yeast library with the human Ad E1B 55-kDa protein can tell us something about possible E1B interactions with human cellular proteins, as originally envisioned. Also promising is the identification of possible E1B 55-kDa protein targets found in the same intranuclear location, discrete subdomains within in the nucleus called PODs (Doucas et al., 1996). The E1B-interacting protein DAXX is found in PODs (Ishov et al., 1999; Li et al., 2000a), associated with SUMO-1-modified PML (Torii et al., 1999).

As a result of adenovirus infection PODs undergo a dramatic morphological change from spherical structures to fibrous-like tracks (Doucas et al., 1996). The reorganization of PODs may be a consequence of infection. However, several viruses express proteins that specifically modify PODs such as herpesviruses: CMV, HSV-1, and EBV (Müller and Dejean, 1999; Adamson and Kenney, 2001). PODs are disrupted in APL linking these nuclear bodies to normal cell growth and development (reviewed in

Melnick and Licht, 1999). There is also evidence of a role for PODs in the control of gene expression (Zhong et al., 2000c) as suggested by the protein components of these structures, including p53, pRb, and CBP. The major component of PODs, PML, is induced by type I interferon (Lavau et al., 1995). It is a regulator of major histocompatibility complex class I presentation (Zheng et al., 1998) and necessary for multiple apoptotic signals (Wang et al., 1998). Taken together it looks as if PODs have antiviral potential and dismantling these nuclear bodies is likely a viral strategy to assure replication. Overexpression of PML effectively suppresses Ad5 E1A/E1B-mediated transformation (Nevels et al., 1999). This suggests that modulation of PML and/or POD components plays a role in transformation by adenovirus E1 gene products. Doucas et al. (1996) reported that E4orf3 11-kDa was sufficient for POD redistribution in Hep-2 carcinoma cells. The E4orf3 11-kDa protein, however, does not antagonize the growth suppressing activity of PML (Nevels et al., 1999). E1B 55-kDa itself localizes to PODs shortly after adenovirus infection, along with E4orf3 (Doucas et al., 1996). To test whether the expression of Ad E1B 55-kDa affects POD formation, the intracellular localization of PML was examined in three cell lines: G401, a rhabdoid kidney tumor cell line, G401 CC3, a derivative of G401 expressing Ad12 E1B 55-kDa protein, and 293, human embryonic kidney cells expressing Ad5 E1A and E1B proteins. Figure 9 illustrates the extensive dispersion of PODs in G401 CC3 and 293 cells. This indicates that Ad E1B 55-kDa proteins are capable of rearranging PODs in the absence of E4orf3. Apart from altered POD formation PI nuclear staining revealed that a fraction of PML proteins are relocalized to the cytoplasm as a result of Ad12 E1B 55-kDa expression (figure 10B and 10C).

This research on E1B 55-kDa and PODs stemmed from the discovery of a potential association with POD component DAXX from the Hela cDNA library. Yeast two-hybrid assays were performed to map the domains responsible for the interaction (figure 8). From the results we can conclude that the C-terminal domains of both Ad 2 E1B 55-kDa and E1B of highly oncogenic Ad 12 interact with the C-terminus of DAXX. The DAXX/Ad12 E1B 55-kDa interaction could not be verified by immunofluorescence microscopy. Protein co-localization was examined in the G401 CC3 cell line (figure 11). With both proteins dispersed throughout the nuclei it is difficult to say whether or not there is co-localization between Ad12 E1B 55-kDa and DAXX when the two images are merged (figure 11C). Work on this project has been ongoing and it has been established that DAXX and E1B 55-kDa interact in vitro and in vivo (Colosimo et al., in preparation). Two distinct regions of DAXX, centered around aa 500-574 and 621-740, interact with the E1B protein in two-hybrid assays. The identification of a DAXX/Ad E1B 55-kDa interaction has interesting implications for the role of E1B in cell transformation. DAXX is thought to mediate Fas ligand-induced apoptosis in response to various stimuli (Torii et al., 1999). This introduces a new link between the adenovirus oncoprotein and prevention of programmed cell death.

Torii et al. (1999) showed that the pro-apoptotic activity of DAXX is dependent on a DAXX-PML interaction. Given that E1B could bind the C-terminus of DAXX, a region responsible for the association of DAXX with PML and the localization of DAXX to PODs (Zong et al., 2000b; Torii et al., 1999), the next step was to examine the effects of Ad E1B 55-kDa expression on PML/DAXX colocalization. Two-color

immunostaining experiments were performed. Endogenous labeling patterns of PML and DAXX were compared in G401, G401 CC3, and 293 cells (figure 9). An altered staining pattern is seen for both proteins. PML/DAXX colocalization was disrupted in the presence of Ad5/12 E1B 55-kDa proteins. Contrary to the effect that E1B protein expression has on PML intracellular localization, DAXX remains in the nucleus in G401 CC3 and 293 cells. This may be pertinent considering new findings by Ko et al. (2001) that the cytoplasmic localization of DAXX is necessary for Fas-mediated apoptosis. A proapoptotic kinase, ASK1, sequestered DAXX in the cytoplasm and inhibited the repression of basal transcription by DAXX.

The combined data implies that another mechanism by which Ad E1B 55-kDa contributes to cellular transformation might be by preventing DAXX-induced apoptosis. This could ensue from interfering with PML action on DAXX and/or sequestering DAXX in the nucleus. Further studies arising from these results suggest that E1B inhibits apoptosis by abrogating both PML and p53 regulation of DAXX while enhancing DAXX-mediated transrepression (Colosimo et al., in preparation). DAXX acts as a potent transcriptional repressor (Hollenbach et al., 1999; Torri et al., 1999). PML inhibited transcription repression by DAXX (Li et al., 2000a) but could not do so in the presence of E1B 55-kDa. p53 (aa 253-393) was found to interact with DAXX (aa 500-574). It relieved DAXX-mediated transrepression but, as with PML, not when co-expressed with E1B 55-kDa. E1B 55-kDa is a powerful transcription repressor (Yew et al., 1994) and strengthens the ability of DAXX to repression transcription. It is known to bind HDAC1 (Punga and Akusjärvi, 2000). These studies also reveal that both DAXX

and E1B bind mSin3A and DAXX binds HDAC1, therefore the DAXX repression complex may be enhanced by the recruitment of additional corepressors and HDACs by E1B 55-kDa (Colosimo et al., in preparation).

Research prospects extending from these results are numerous. Regarding DAXX, future experiments could test whether E1B 55-kDa competes with PML for DAXX binding. E1B may also interfere with the binding of the C-terminal region of DAXX to ETS1. ETS1 is one of the cellular genes that make up the transforming protein of the E26 virus. When DAXX is bound to ETS1 it represses ETS1 dependent transactivation of the BCL2 and MMP1 promoters (Li et al., 2000b). BCL-2 is an anti-apoptotic protein while MMP1 is involved in the process of tumor invasion and metastasis. Whether these genes are expressed in G401 CC3 and 293 cells can be determined. DAXX also represses Pax3 transcriptional activity (Hollenbach et al., 1999). Pax3 is fused to transcription factor FKHR in alveolar rhabdomyosarcoma, a malignant tumor of skeletal muscle, as a result of the t(2;13) (q35;q14) translocation (Shapiro et al., 1993; Galili et al., 1995). Interestingly, the oncogenic fusion product is no longer regulated by DAXX (Hollenbach et al., 1999). It could be tested whether or not E1B 55-kDa interferes with the regulation of Pax3 by DAXX. A look into what genes are regulated by DAXX would give us more information on the significance of the DAXX/E1B interaction. Also unknown is the region(s) of E1B 55-kDa required for POD dispersal. Various E1B mutants could be expressed in G401 cells and resulting protein localization examined. This might also tell us whether the DAXX/E1B interaction was important for disruption of PODs. In the absence of PML, DAXX is located in areas of

condensed chromatin (Ishov et al., 1999). It would be interesting to determine if it is associated with condensed chromatin in the cell lines expressing E1B 55-kDa proteins. Aside from DAXX, library screening has provided a number of potential E1B-interacting proteins for consideration. With the exception of Sumo-1 and UbcH7, they remain to be confirmed by alternate means. Proteins that look promising and will be discussed here include UFD1, UbcH7, Ub, SUMO-1, Bdf2, Clk1, MCM2, and Yap4/6.

Yeast UFD1, as mentioned above, is involved in the Ub-proteasome pathway, a major pathway for the selective turnover of intracellular proteins in eukaryotes, along with human UbcH7 and Ub itself. This pathway regulates many cellular processes by controlling the levels of key regulatory proteins such as cyclins, histones, oncoproteins and tumor suppressors. It is possible that E1B itself is targeted for degradation by Ub conjugation. The direct interaction between E1B 55-kDa and UbcH7ΔE2 was verified in vitro by immunoprecipitation using purified protein preparations (figure 7C). This alternatively spliced version of UbcH7 lacking exon 2 has not been previously described, although it may have resulted during construction of the HeLa cDNA library. UbcH7 is the Ub-conjugating enzyme required for Ub-mediated degradation of the tumor suppressor protein, p53, normally short-lived in cells. The level of p53 in 293 and G401 CC3 cells expressing Ad E1B 55-kDa proteins is markedly increased (data not shown). The mechanism by which E1B stabilizes p53 is not known but one mechanism may be by interfering with p53 proteolysis through its interaction with UbcH7. Other viral proteins, such as the transforming proteins of HPV-16 and HPV-18, enhance Ub-dependent degradation of cellular tumor suppressors. E6 associates with p53 (Werness et al., 1990),

while E7 associates with pRb (Boyer et al., 1996), and they target the proteins for degradation by the Ub-proteasome pathway. E1B 55-kDa may also interfere with the ubiquitination of other proteins requiring UbcH7. It is responsible for Ub-mediated degradation of the oncoprotein c-fos (Stancovski et al., 1995) and processing of the NfκB precursor p105 (Orian et al., 1995). NfκB targets genes involved in cell growth and differentiation, inflammation, lymphocyte activation, and the acute-phase response. A human homologue of UFD1, UFD1L, has been described as a potential housekeeping gene (Novelli et al., 1998). It will be interesting to discover whether Ad E1B 55-kDa interacts with UFD1L.

Hela cDNA library screening also identified a Ub-like protein, SUMO-1, which covalently modifies a limited number of proteins in a manner analogous to ubiquitination (Kamitani et al., 1997). SUMO-1 has not been shown to target proteins for degradation; instead, this modification affects protein activity and/or intracellular location. Given that SUMO-1 was hit upon from screening we thought it possible that E1B itself could be a substrate for sumolation. There are other examples of SUMO-1 modification of viral proteins: Epstein-Barr virus (EBV) immediate-early protein BZLF1 (Adamson and Kenney, 2001) as well as cytomegalovirus (CMV) IE1 (Müller and Dejean, 1999) and IE2 (Hofmann et al., 2000) proteins. In fact, during the preparation of this thesis, Endter et al. (2001) published that Ad5 E1B 55-kDa is modified by SUMO-1 in vivo at lysine residue 104 (K104). This SUMO-1 acceptor site lies within the consensus motif ΨKxE. Sumolation is required for E1B 55-kDa complete transformation by adenovirus and inactivation of p53. It affects both nuclear import and intranuclear localization of the

E1B protein in track- or dot-like structures that resemble reorganized PODs. There are other possibilities that have not been ruled out by the discovery that E1B is sumolated. For example, binding to SUMO-1 could alter the sumolation status of cellular proteins. A decrease in the amount of SUMO-1 modified forms of PML occurs as a result of herpes simplex virus (HSV) ICP0 and CMV IE1 expression and this affects POD formation (Müller and Dejean, 1999). Along the same lines E1B may interfere with sumolation of p53. SUMO-1 modification of p53 has been reported to result in an increase in p53 transactivation activity (Gostissa et al., 1999; Rodriguez et al., 1999). The E4orf3 protein does not interfere with the conjugation of SUMO-1 to PML. There is also the possibility that E1B binds to SUMO-1 modified proteins through non-covalent interaction or that SUMO-1 provides an additional interaction motif for cellular factors.

Future studies should include investigating the interaction between E1B 55-kDa and Bdf2. Bdf2, functionally redundant with Bdf1, contains two copies of the evolutionarily conserved bromodomain, a 110 amino acid module. The bromodomain is ubiquitously found in eukaryotes, in proteins implicated in diverse functions. For this reason it is difficult to ascertain the utility of this domain. Many bromodomain-containing proteins participate directly in chromatin modification and transcription regulation. Nearly all of the nuclear HATs contain bromodomains, including P/CAF (Yang et al., 1996), p300/CBP (Bannister and Kouzarides, 1996; Ogryzko et al., 1996), and $TAF_{II}250$ (Mizzen et al., 1996). Bromodomains interact specifically with acetylated lysines of histone H4 (Dhalluin et al., 1999; Jacobson et al., 2000; Owen et al., 2000; Hudson et al., 2000). A fragment of Bdf1 that includes one of the two bromodomains has

binding affinity for H3 and H4 (Pamblanco et al., 2001). Given that Bdf1/Bdf2 is homologous to the carboxyl-terminal half of mammalian $TAF_{II}250$ (Matangkasombut et al., 2000) it would be important to find out if E1B 55-kDa targets $TAF_{II}250$ in adenovirus-infected cells. By and large, the bromodomain may be a new E1B-interacting region. E1B 55-kDa binds to another bromodomain-containing protein, P/CAF (Liu et al., 2000). It was not determined whether or not the bromodomain of P/CAF was required for the interaction, however, mutant P/CAF missing residues 62 to 464 but with an intact bromodomain (aa 722-830) could still bind E1B 55-kDa (Liu et al., 2000). This interaction prevents P/CAF from binding to p53 thereby inhibiting acetylation of p53 by P/CAF (Liu et al., 2000). Interfering with the functions of bromodomain-containing proteins may have wide spread consequences. The specific loss of the p300 bromodomain in the human cervical carcinoma cell line SiHa leads to impairment of p300 coactivator function and implicates the bromodomain in tumor suppressor activity of p300 (Ohshima et al., 2001). Physical interaction with the bromodomain could target E1B 55-kDa directly to chromatin or chromatin-associated complexes. Considering these observations, an important future experiment would be to determine if the bromodomain is a newly discovered E1B-interacting domain. This would lead to an explosion of data on E1B 55-kDa and its role in adenovirus-mediated cellular transformation

Other proteins that it may be interesting to investigate further include cdc2-like kinase, Clk1, also termed STY. It was the first dual specificity kinase to be discovered, so called because they can phosphorylate both serine/threonine and tyrosine residues (Ben-David et al., 1991; Howell et al., 1991; Johnson and Smith, 1991). It contains a C-

terminal catalytic domain and an N-terminal functional nuclear localization domain (Duncan et al., 1995). The N-terminus also encodes a regulatory domain (Menegay et al., 2000). Clk1 affects the splicing of mRNA as a result of the phosphorylation of SR proteins (Duncan et al., 1997; Colwill et al., 1996). It can regulate its own splicing as well as the splicing of Ad E1A pre-mRNA (Duncan et al., 1997). It is possible that this kinase is involved in altering the mRNA splicing patterns during the switch from early to late phase of adenovirus replication. An interaction with Clk1 would implicate E1B 55-kDa in this process. There is also a mini-chromosome maintenance protein, MCM2. MCM2 is required for initiation of DNA replication (Todorov et al., 1994). In all eukaryotes MCM proteins 2-7 ensure that DNA replicates only once in each cell cycle (for a review on MCM2-7 see Tye, 1999). MCM2 was found to associate with an EBV replication origin, *oriP* along with the human origin recognition complex, ORC, in a cell cycle dependent manner (Chaudhuri et al., 2001). An interaction between E1B 55-kDa and MCM2 would point towards a possible involvement of these proteins in adenovirus DNA replication. Yap4 and Yap6 were found to bind to Ad2 E1B 55-kDa in yeast two-hybrid assays. Yaps are bZIP proteins reminiscent of human AP-1 transcription factors (Fernandes et al., 1997). Yaps are related in the coiled coil leucine zipper present in the bZIP DNA-binding domain, and in DNA-binding specificity, distinct from AP-1 factors. Unlike other Yaps, Yap4 and Yap6 share sequence similarity in the leucine zipper. Since this domain is present in both fragments obtained by screening perhaps it is targeted by the E1B 55-kDa protein. Lastly, the metastasis-associated *MTA1* gene encodes a nuclear protein product of unknown function. Expression of this gene has been correlated with the metastatic potential of gastrointestinal and breast carcinomas although it is also

expressed in many normal tissues (Nawa et al., 2000; Toh et al., 1997). Association with MTA1 encourages investigation as there is evidence for the involvement of MTA1 in the process of cellular proliferation. Inhibition of *MTA1* gene expression by antisense phosphorothioate oligonucleotides abrogated cell growth of MDA-MB-231 breast cancer cells. Also, the MTA1 protein physically associates with HDAC1 (Toh et al., 2000) and thus may be active in chromatin remodeling. Possible E1B 55-kDa effects on MTA1 would tell us more about the role MTA1 plays in cell proliferation and metastasis.

The goal of elucidating the role of the E1B 55-kDa oncoprotein in adenovirus transformation is to be able to better understand the progression of cancer and cancer cell growth. Human cells become malignant by a multistep process. Prior to neoplastic transformation, cells must first become immortalized. Overcoming aging is a critical step in malignant tumor formation. Many types of human cells can be immortalized by adenovirus oncogenes (Byrd et al., 1982). Compound genetic changes are required to induce immortalization. Oncoproteins of DNA tumor viruses, including Ad E1B 55-kDa, SV40 LT, and HPV E6, inactivate p53. The result of p53 inactivation is genomic instability leading to additional chromosomal aberrations (reviewed in Shay et al., 1991). Adenovirus infection is not associated with any known cancers. However, investigating the mechanisms of immortalization by adenovirus oncogenes can further our understanding of the multistep process of carcinogenesis. Once cells become immortalized by adenovirus they are readily made neoplastic by a variety of agents (reviewed in Rhim et al., 1990). Although all adenovirus serotypes are capable of immortalizing cells in tissue culture, the oncogenic potential of each is different. Highly

oncogenic Ad12 can induce malignant tumor formation in rodents (Trentin et al., 1962). To perform the yeast two-hybrid screen we had originally intended to use the E1B 55-kDa protein of Ad12. When we experienced problems cloning the construct into the pGBDU plasmid we began working with the present bait. When several Ad12 E1B deletion mutants were obtained and expressed as Gal4 BD fusion proteins we realized that they had negative or detrimental effects on yeast cell growth to varying degrees. We obtained fewer colonies following transformation and these colonies were smaller in size than expected. This would make screening the expression libraries an arduous task. Many of the potential E1B-binding partners were tested in yeast two-hybrid assays for interaction with Ad12 E1B 55-kDa with positive results (figure 8 and data not shown). With the focus on E1B-binding proteins involved in cell cycle regulation or apoptosis to further investigate Ad E1B 55-kDa action in cell transformation, of particular interest was the interaction between DAXX and Ad2/12 E1B 55-kDa. Preventing DAXX-mediated apoptosis may be another mechanism by which the E1B oncoprotein perturbs cell growth regulation. Modulation of DAXX and PML functions may be required for complete transformation of cells by E1B 55-kDa in cooperation with E1A. This research has also suggested further possibilities by which Ad E1B 55-kDa inhibits p53 function. It has been proposed that E1B 55-kDa may couple a direct transcriptional repressor to p53 (Martin and Berk, 1998). Also, E1B 55-kDa specifically inhibits acetylation of p53 by PCAF (Liu et al., 2000). Disrupting PODs may be one mechanism by which E1B prevents p53 activation. The interaction between p53 and PML and the consequent targeting of p53 to PODs leads to an increase in p53 transcriptional activity and affects cell survival (Fogal et al., 2000). Disrupting PODs would also disrupt the p53/DAXX

interaction suggested by Fogal et al. (2000). This research launched a series of experiments that suggest E1B 55-kDa enhances DAXX-mediated transrepression and prevents both PML and p53 regulation of DAXX (Colosimo et al., in preparation). Thus insight has been gained into the workings of PML oncogenic domain components and into relationships between the cellular tumor suppressor proteins, PML and p53, with DAXX.

Appendix A – Yeast and Bacteria Media

Yeast Selective Dropout Media

Ingredients	Quantity for 1L
Yeast Nitrogen Base (without amino acids)	1.7 g
Ammonium Sulfate	5.0 g
Dextrose	20 g
Amino Acid Solution*	10 ml
Essential amino acids: Adenine (2 mg/ml)	10 ml
Histidine (10 mg/ml)	2 ml
Leucine (10 mg/ml)	3 ml
Lysine (10 mg/ml)	3 ml
Tryptophane (10 mg/ml)	2 ml
Tyrosine (0.75 mg/ml)	40 ml
Uracil (2 mg/ml)	10 ml

- Any of the 7 essential amino acids can be left out
- Adjust the pH between 5.0 and 6.0
- For SD agar add 16.6 g of agar for 1 L
- Autoclave

*Amino Acid Solution

Ingredients	Quantity
Arginine	0.4 g
Methionine	0.4 g
Isoleucine	0.6 g
Phenylalanine	1 g
Glutamic acid	2 g
Aspartic acid	2 g
Valine	3 g
Threonine	4 g
Serine	8 g
H_2O	200 ml

- Heat in the microwave for 1-2 min.
- Autoclave

Stock Preparation for Essential Amino Acids

Ingredients	Quantity in 100 H_2O	Result	Storage Temperature
Adenine	0.2 g	2 mg/ml	Room Temperature
Histidine	1.0 g	10 mg/ml	4^0C
Leucine	1.0 g	10 mg/ml	Room Temperature
Lysine	1.0 g	10 mg/ml	4^0C
Tryptophane	1.0 g (heat)	10 mg/ml	4^0C
Tyrosine	0.075 g (heat)	0.75 mg/ml	Room Temperature
Uracil	0.2 g	2 mg/ml	Room Temperature

- Autoclave

Bacterial YT Media

Per liter:

8 g Bacto-tryptone
5 g Bacto-yeast extract
2.5 g NaCl

Appendix B - List of Solutions

De-stain solution
15% ethanol
5% acetic acid

Dialysis buffer
50 mM Tris-HCl, (pH 8.0)
10% glycerol
1 mM DTT
1 mM PMSF
0.1 mM EDTA

His binding buffer
0.5 M NaCl
20 mM Tris-HCl (pH 7.9)
20 mM imidazole

His elution buffer
0.5 M NaCl
20 mM Tris-HCl (pH 7.9)
200 mM imidazole

His wash buffer
0.5 M NaCl
20 mM Tris-HCl (pH 7.9)
60 mM imidazole

LETS buffer
0.01 M Tris-HCl, (pH 8.0)
0.1 M LiCl
0.01 M EDTA
0.2 % SDS

LiOAc solution
0.1M LiOAc
10mM Tris-HCl
1mM EDTA, (pH 8.0)

SDS gel-loading buffer
50 mM Tris-Cl (pH 6.8)
100 mM dithiothreitol
2% SDS
0.1% bromophenol blue
10% glycerol

0.1M Sodium phosphate buffer (pH 7.0)
57.7 mM Na_2HPO_4
42.3 mM NaH_2PO_4

Solution I
50 mM glucose
25 mM Tris-Cl (pH 8.0)
10 mM EDTA

Solution II
0.2 N NaOH
1% SDS

Solution III
3 M potassium acetate
11.5% glacial acetic acid

TAE
0.04 M Tris-acetate
0.1 M EDTA

TE
10 mM Tris-Cl (pH 8.0)
1mM EDTA

Transfer buffer
20 mM Tris
50 mM glycine
20% methanol

Tris-glycine electrophoresis buffer
25 mM Tris
250 mM glycine
0.1% SDS

Wash buffer 1
50 mM Tris-Cl (pH 7.5)
0.5 M NaCl
0.1% NP-40
1 mM EDTA
0.25% gelatin
0.02% sodium azide

Wash buffer 2
50 mM Tris-Cl (pH 7.5)
0.5 M NaCl
0.1% NP-40
1 mM EDTA
0.25% gelatin
0.02% sodium azide
0.1% SDS

Wash buffer 3
10 mM Tris
0.1% NP-40

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